BARD Final Report US-4599-13R

The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aqua-feeds

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Project award year: 2013
Three year research project

ABSTRACT: The FAO has projected a doubling in world demand for seafood during the 21st century, and that most of the needed product would be supplied from aquaculture of marine fish and shrimps fed primarily on fishmeal-based aquafeeds. However, current practices of high intensity monoculture of shrimp in coastal ponds and fish in offshore pens have been strongly criticized as being ecologically and socially unsustainable. This view derives from un-checked eutrophication of coastal marine ecosystems from fish farm effluents, and the destruction of coastal estuarine ecosystems by shrimp farm constructions, plus aquaculture's reliance on wild-caught small fish - which are excellent food for humans, but instead are rendered into fishmeal and fish oil for formulating aquafeeds. Fishmeal-sparing and waste-reduction aquafeeds can only delay the time when fed aquaculture product are priced out of affordability for most consumers. Additionally, replacement of fishmeal protein and fish oil by terrestrial plant sources such as soybean meal and oil directly raises food costs for human communities in developing nations. New formulations incorporating sustainably-produced marine algal proteins and oils are growing in acceptance as viable and practical alternatives. This BARD collaborative research project investigated a sustainable water-sparing spray/drip culture method for producing high-protein marine macrophyte meals for incorporation into marine shrimp and fish diets. The spray culture work was conducted at laboratory-scale in the USA (UCSD-SIO) using selected *Gracilaria* and *Ulva* strains isolated and supplied by UCONN, and outdoors at pilot-scale in Israel (IOLR-NCM) using local strains of *Ulva* sp., and nitrogen/phosphorus-enriched fish farm effluent to fertilize the spray cultures and produce seaweed biomass and meals containing up to 27% raw protein (dry weight content). Auburn University (USA) in consultation with TAMUS (USA) used the IOLR meals to formulate diets and conduct marine shrimp feeding trials, which resulted in mixed outcomes, indicating further work was needed to chemically identify and remove anti-nutritional elements present in the IOLR-produced seaweed meals.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Abstract	0	0	1
Abstract - Poster	0	0	1
Book Chapter	1	0	0
Review Article	1	0	0
Reviewed	1	1	4
Submitted	0	2	1
Thesis - MSc.	1	0	0
Thesis - Ph.D.	0	0	1

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
M.Sc. Student	Bronfman	Y.	Hebrew University of Jerusalem	Israel
Ph.D. Student	Qiu	Xuan	Auburn University	USA

Details of Cooperation between the Partners to Attain Project Objectives:

The project team consisted of senior marine science researchers, Ph.D. and Masters students, and technicians from four USA universities (UCSD-SIO, TAMUS, UCONN, AUBURN), and one Israeli research institute (IOLR-NCM). Dr. B. Greg Mitchell, Research Biologist at UCSD-SIO acted in capacity as the overall project Principal Investigator (P.I.), assisted by Dr. D. Mendola, Senior Development Engineer at UCSD-SIO for overall project management and coordination. At UCONN, Dr. Charles Yarish, Professor at the Department of Ecology & Evolutionary Biology, assisted by Dr. Jang K. Kim, Assistant Research Professor and technicians and undergraduate students, identified and isolated seaweed species and strains suitable for the spray/drip system, and provided biomass of the selected seaweed species to project partners. At TAMUS, Dr. Tzachi Samocha acted as P.I. and resident marine shrimp biology and aquaculture specialist, assisted by shrimp nutritionist Dr. Anthony Saccardi. At AUBURN, Dr. D. Allen Davis assisted by Dr. X. Qiu and their graduate students and technicians, designed and directed the marine shrimp feeding trials using high-protein seaweed meals supplied by IOLR-NCM, which was under the co-direction of Drs. A. Neori and L. Guttman, with assistance from Dr. M. Shpigel, and their graduate students and research technicians.

Communications between project partners was primarily via e-mail; however, telephone communications was used on a regular basis between USA collaborators, and on occasion with IOLR collaborators and USA partners. Project administration was managed by UCSD-SIO Grants and Contract Officer, Mrs. Ann Dunbar, who maintained regular contact with BARD administrators, mostly via e-mail communications.

Materials were sent between partners under Material Transfer Agreements, and via commercial courier services.

Major Achievements of the Project: US-4599-13R

(1) Project collaborators at UCONN (USA) isolated and identified a number of marine macrophyte ("seaweed") species and strains (from the "red" and "green" species lineages), that possessed the necessary biological and growth characteristics to be adaptable to the spray/drip system of irrigation, and the warmer culture temperatures of a greenhouse-heated environment;

(2) Project collaborators at UCSD-SIO (USA) conducted a series of laboratory-scale spray/drip culture experiments to evaluate the effectiveness of media formulations mimicking the nitrogen and phosphorus concentrations of typical marine fish farm effluents on growth, biomass productivity and total protein content of the red and green seaweed strains provided by UCONN collaborators;

(3) Israel collaborators (IOLR-NCM) designed then built a pilot-scale module of a technically simple, energy efficient, spray/drip culture system employing effluent from marine fish ponds for drip irrigation.

(4) The IOLR-NCM collaborators operated the pilot module to grow batches of protein-rich, locally-collected (Mediterranean and Red Sea) strains of red and green marine seaweeds; then solar / air dried the cultured biomass to produce batches of pulverized seaweed meals.

(5) Auburn (USA) collaborators in consultation with TAMU project scientists (USA) first chemically analyzed the nutritional components of the IOLR-produced seaweed meals, then formulated experimental aquafeed diets incorporating varying amounts of seaweed meals, terrestrial plant meals, and plant oils and energy components, for subsequent testing in controlled shrimp feeding trial at AUBURN.

(6) AUBURN (USA) collaborators analyzed the results of the shrimp feeding trials and reported on the digestibility and food conversion efficiency of IOLR meal-formulated shrimp diets comparing the results obtained to AUBURN-formulated control shrimp diets.

(7) Auburn reported that the shrimp feeding trials produced mixed outcomes, indicating further work was needed to chemically identify and remove anti-nutritional elements present in the IOLR-produced seaweed meals.

(8) All collaborators reported their results obtained from the project to BARD, and in addition, all collaborators either co-wrote, or wrote singularly and/or with non-BARD collaborators a number of scientific manuscripts on the results obtained from the project, for submission to respected phycology, aquaculture, and aquaculture nutrition journals for consideration of publication. To date, 8 manuscripts based upon the work and results obtained under this project have been published, and another 4 are currently being reviewed for publication. In addition, one (1) book chapter has been submitted to editors, and two academic theses were completed by students associated with the project, one Master of Science and one Doctor of Philosophy. Three invited presentations were made on the results of the project and to make presentations before international scientific colloquia to inform other scientists and technologists working in the fields of aquaculture and aquatic animal nutrition of the results of this important research.

Changes to the original research plan_US45-99-13R

One major change to the original research plan was made necessary, when near the end of the 2nd year of planned research, the management of the Texas A&M University, AgriLife Research Division decided to close their Flour Bluff Marine Shrimp Research facility. This facility and attendant personnel, included the TAMUS P.I. Prof. Tzachi Samocha, was originally charged - in the final accepted project proposal to BARD, to prepare the marine shrimp feed formulations and conduct the marine shrimp feeding trials.

In preparation for the TAMUS facility closing, Prof. Samocha inquired with a close collaborator of his at Auburn University School of Fisheries and Applied Aquacultures, Prof. D. Allen Davis, if he would like to inherent the third year project budget and tasks from TAMUS, and conduct the feed formulations and shrimp feeding trials at Auburn. Prof. Davis, honorably accepted the responsibility and happily accepted the third year budgetary set-aside that was originally due to TAMUS, and after some time with administrative necessities between the two universities, the transfer was successfully completed, and the 3rd year research was begun and completed at Auburn.

Publications for Project US-4599-13R

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Gorman, L., Kraemer G.P., Yarish C., Boo S.M. and Kim J.K.*	The effects of temperature on the growth and nitrogen content of invasive Gracilaria vermiculophylla and native Gracilaria tikvahiae from Long island Sound, USA.	***Algae***	32 : 57:66 2017	US only
Published	Reviewed	*Kim, J.K. and Yarish C.*	Development of a sustainable land-based Gracilaria cultivation system	***Algae***	29 : 217:225 2014	US only
Published	Reviewed	*Zhang, J., Kim J.K., Yarish C. and He P*	The expansion of Ulva prolifera O.F. M?ller macroalgal blooms in the Yellow Sea, PR China, through asexual reproduction.	***Marine Pollution Bulletin***	104 : 101:106 2016	US only
Published	Reviewed	*Levy, A., Neori, A., Harpaz, S., Shpigel, M. & Guttman, L.*	Marine periphyton biofilters in mariculture effluents: nutrient uptake and biomass development	***Aquaculture***	473 : 513-520 2017	IS only
Submitted	Reviewed	*Qiu, X., A. Neori, J. Kim, C. Yarish,M. Shpigel, L. Guttman, D. Ben-Ezra, V. Odintsov, D. A. Davis*	Evaluation of green seaweed Ulva sp. as a replacement of fish meal in plant-based practical diets for Pacific white shrimp, Litopenaeus vannameiEvaluation of green seaweed Ulva sp. as a replacement of fish meal in plant-based practical diets for Pacific white shrimp, Litopenaeus vannamei	***Journal of Applied Phycology***	:	Joint
Published	Review Article	*Neori, A., Shpigel, M., Guttman, L., Israel, A*	The development of polyculture and integrated multi -trophic aquaculture (IMTA) in Israel: A review	***Isr. J. Aquacult. - Bamidgeh***	69 : 2017	IS only
Submitted	Reviewed	*Qiu, X., A. Neori, J. Kim, C. Yarish,M. Shpigel, L. Guttman, D. Ben-Ezra, V. Odintsov, D. A. Davis*	Green seaweed Ulva sp. as an alternative ingredient in plant-based practical diets for Pacific white shrimp, Litopenaeus vannamei.		: 2017	Joint
Published	Thesis - Ph.D.	*Qiu, Xuan*	ingredients in practical diets for Pacific white shrimp (Litopenaeus vannamei)		: 2017	US only
Published	Abstract	*Gorman L., Kim J.K, Yarish C. and Kraemer G.*	The effect of temperature on growth of non-native seaweed species Gracilaria vermiculophylla in the Long Island Sound as compared to native Gracilaria tikvahiae.	***SUNY Undergraduate Research Conference***	: 2015	US only
Published	Thesis - MSc.	*Bronfman, Y.*	The use of aquaculture effluents in spray culture for the production of high protein	***The Hebrew University of Jerusalem***	: 2016	IS only

			macroalgae for shrimp aqua-feeds			
Published	Abstract - Poster	*Mendola, W.G., D. Mendola, P. Abelin, B.G. Mitchell*	Growth performance of Gracilaria vermiculophylla for three different re-circulating seawater spray system designs	***, Algae Biomass Summit, San Diego, CA, USA***	: 2014	US only
Published	Reviewed	*Kim, J.K., Yarish C., Hwang, E.K., Park, M., and Kim, Y.*	Seaweed aquaculture cultivation technologies, challenges, and its ecosystem services.	***Algae***	32 : 1-13 2017	US only
Submitted	Reviewed	*Mendoza, W.G., D. Mendola, J. Kim, C. Yarish,A. Velloze, B.G. Mitchell.*	Development of drip irrigation methods for land-based biomass and protein production of Ulva compressa.	***PLosONE***	:	US only
Published	Book Chapter	*Neori, A., Shpigel, M. and Israel, A.*	The development of integrated multi -trophic aquaculture (IMTA) in Israel.	***Greening the Blue Revolution: the Turquoise Revolution of Integrated Multi-Trophic Aquaculture (IMTA), T. Chopin, A. Neori, S. Robinson and M. Troell (Eds.), Springer Publishers, Dordrecht.***	: 2017	IS only
Published	Reviewed	*T.M. Samocha a, J. Fricker a, A.M. Ali b, M. Shpigel c, A. Neori*	Growth and nutrient uptake of the macroalga Gracilaria tikvahiae cultured with the shrimp Litopenaeus vannamei in an Integrated Multi-Trophic Aquaculture (IMTA) system	***Aquaculture***	446 : 263-271 2015	Joint

Final Scientific Report
Cover Page

BARD Project Number: US-4599-13R

Date of Submission of the Report: June 30, 2017

Project Title: **The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aquafeeds.**

Investigators:	**Institutions**:
Principal Investigator (PI) :	
Mitchell, Brian Gregory	University of California, San Diego, Scripps Institution of Oceanography
Co-Principal Investigator (PI)	
Neori, Amir	Israel Oceanographic and Limnological Research, National Center for Mariculture, Eilat
Collaborating Investigators:	
Yarish, Charles	University of Connecticut
Samocha, Tzachi	Texas A&M University, AgriLife Research
Davis, D. Allen	Auburn University, Department of Fisheries and Allied Aquacultures

Keywords: (no additional keywords)

Abbreviations: (see Page 3 of report)

Budget: (Israel) $108,000 (US) $222,000 (Total) $330,000

Signature:
Principal Investigator

Signature:
Authorizing Official, Principal Institution

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Final Scientific Report

Publication Summary (numbers):

Published	Joint IS/US (1) US Only (5) IS only (2) Total = (8)
Submitted, in-review, in-prep	Joint IS/US (2) US Only (1) IS only (1) Total = (4)
Book Chapters	Joint IS/US (0) US Only (0) IS only (1) Total = (1)
Masters Theses	Joint IS/US (0) US Only (0) IS only (1) Total = (1)
Ph.D. Theses	Joint IS/US (0) US Only (1) IS only (0) Total = (1)

Postdoctoral Training: No postdoctoral researchers received more than 50% of their funding from the BARD grant.

Cooperation Summary (numbers)

	US-to-Is	Is-to-US	Together / elsewhere	
Short Visits:	(2)	(0)	(2)	Total = (4)

Description of the Cooperation:

During the first year of performance on the project, Dr. Mitchell (Overall project P.I. at UCSD-SIO) was assisted by Dr. Mendola to maintain close e-mail communication with all BARD project partners, and regular telephone contact with Professors Yarish & Jang (UCONN), and Prof. Samocha at TAMU. Close coordination has been maintained between UCONN & UCSD-SIO to facilitate transfer of *Gracilaria* spp. algal biomass from UCONN to SIO required for SIO's lab-scale spray culture experiments. In Israel, Dr. Neori's work in the second year was significantly delayed due to lasting civil unrest in Israel, and the real and daily dangers of working out of doors at the National Center for Mariculture in Eilat, and elsewhere in the country. Despite the near daily assaults, Dr. Neori managed to guide the construction of the experimental array for spray culture experiments, and complete a number of the planned Yr-1 experiments. During these trying times, e-mail communication was maintained between each of the USA-partners and Dr. Neori, both for moral support for him and his family, and for maintaining technical discussions on the design and implementation of his system and the planned experiments.

In early October, 2014, Prof. Tzachi Samocha (TAMU) traveled to Israel to visit with Dr. Amir Neori (NCM) to discuss the initial results from Dr. Neori's experiments. Prof. Yarish (UCONN) talked with Prof. Samocha in anticipation of his trip to Israel to suggest that he convey to Dr. Neori that he should consider increasing the nitrogen content of his spray culture media, based upon encouraging results from then published work of UCONN. Drs. Yarish and Kim visited UCSD / Scripps Institution of Oceanography as part a bilateral program between NOAA and The *National Fisheries Research and Development Institute* (*NFRDI*) of Korea on Mar. 23rd, 2015. Drs. Yarish and Kim discussed seaweed cultivation systems and experimental design for the project with project PI, Drs. B. Greg Mitchell and Dr. Dominick Mendola, as well as SIO's post-doctoral BARD researcher, Dr. Wilson Mendoza. Dr. Yarish also visit IOLR and NCM, Haifa and Eilat, Israel, with the support of The UCONN Office of Global Affairs (Jan. 7-9, 2015). During this trip, Dr. Yarish delivered UCONN's *Ulva compressa* (U-CC-ST1) and *Gracilaria* vermiculophylla (G-NY-ST4 and GV-KR-ST1) strains to Israel Oceanographic and Limnological Research Institute and The National Center for Mariculture (both at Haifa and Eilat) and made presentations at our partner institutions. All-in-all and throughout the entire 3-year project period – and the ca. 1-year no-cost extension, communications were very well maintained between all project partners, and proved to be crucial to keeping the project moving forward, and progressing the aims and scientific objectives to an overall productive conclusion of the research in March, 2017.

Patent Summary (numbers): NONE

Abbreviations used in this report:

AA - Amino acids
ADC – Apparent digestibility coefficient(s)
ADAA – Apparent digestibility coefficient of amino acids
ADE – Apparent digestibility coefficient of energy
ADMD – Apparent digestibility coefficient of dry matter
ANOVA – Statistical analysis of variance
AUBURN – University of Auburn, Department of Fisheries and Allied Aquacultures
ArA - Arachidonic acid
Chl a – Chlorophyll a
Chl b – Chlorophyll b
CHN – Carbon, hydrogen and nitrogen content
C, N, P – Carbon, nitrogen and phosphorus (respectively) nutrients
CT – State of Connecticut
DHA – Docosahexanoic acid
dw – Dry Weight
EPA – Eicosapentanoic acid
FAO –Food and Agriculture Organization of the United Nations
FCR – Food conversion ratio (weight of consumed food divided by live weight gain)
IMTA– Integrated Multi-Trophic Aquaculture
IOD – Integrative Oceanography Division at UCSD-SIO (Dr. Mitchell's division)
IOLR-NCM – Israel Oceanographic and Limnological Research, National Center for Mariculture, Eilat (and/or IOLR, or NCM)
JSM – Jack's Special Media
LIS – Long Island Sound
NH_4^+ – Total ammonia (NH_4^+ + NH_3)
NO_2^- – Nitrite
NO_3^- – Nitrate
PO_4^{-3} – Orthophosphate
PAM – Pulse Amplitude Modulation
PR – Apparent net protein retention
PT – Portugal
QC'd – Quality controlled
TAMUS –Texas A & M University System, Texas AgriLife Research
RI – State of Rhode Island, USA
TAN – Total ammonia nitrogen
TSS – Total suspended solids
UCONN – University of Connecticut
µM – micro-mol
UCSB – University of California, Santa Barbara
UCSD-SIO – University of California San Diego, Scripps Institution of Oceanography
UCONN – University of Connecticut
VSE – von Stosch enriched medium

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APPENDIX - unpublished results and data obtained during the BARD-sponsored, 3-year research project.

YEAR 1 – Research Results

1) Partner Organization: University of Connecticut (UCONN)

Task / Objectives: Collect and Screen red and green seaweed candidates from several genera including *Gracilaria* and *Ulva* for temperature tolerance and growth under spray cultivation conditions.

Introduction: *Gracilaria*: During previous projects supported by EPA Long Island Sound Futures Fund (NFWF/Legacy Grant Project ID: 1401.10.024266), CT Sea Grant (R/A 38, NA10OAR4170095), Woods Hole Sea Grant College Programs (NA10OAR4170083) and the U.S. Department of Energy's NETL Program (FOA #0000015), over 10 strains of the *Gracilaria tikvahiae and G. vermiculophylla* species from CT, RI, MA and NY were isolated and continue to be maintained at UCONN. During the first year of the BARD project, we isolated an additional strain of *G. vermiculophylla* from Qingdao, China. Species of all strains was determined by DNA sequencing using ribosomal small-subunit 18s RNA. Additionally, *G. vermiculophylla*, collected from east coast of Korea, which is a haplotype of all invasive strains of this alga in Europe and North America, was also transferred from Prof. S.M. Boo's laboratory (Chungnam University, Korea) to UCONN's laboratory.

We have established and propagated vegetative cultures of male and female gametophytes and tetrasporophytes, isolated from spores. We also established cultures initiated from field collected plants through vegetative propagation. In both cases, clean plants in good condition were collected from the field and cleaned by gently wiping with sterile cotton balls. Vegetative branches were dragged through seaweed agar to remove epiphytes. For vegetative cloning, the excised vegetative tips were transferred to von Stosch's enriched seawater (VSE) in 50 mm sterile Petri dishes and maintained at 20°C, 20 μmol m^{-2} s^{-1} light and day neutral (12:12, L:D) conditions (Ott, 1965). Tetrasporic and cystocarpic branches were transferred to sterile culture dishes containing VSE and were maintained at 20°C, 10 μmol m^{-2} s^{-1} light and day neutral conditions until spores have been released and begin to germinate. Individual

germlings were transferred to VSE in 50 mm sterile culture dishes and maintained at 20°C, 20 μmol m^{-2} s^{-1} light and day neutral conditions.

Due to the limited time to prepare the *seedstock* for experiments, we selected a fast growing strain of *Gracilaria vermiculophylla* (GV-NY-ST1). Beginning in August, 2013, we began to mass culture this strain in volumes ranging from 1-200L, at UCONN's Stamford, CT laboratory, and also at the BRASTEC facility (UCONN's collaborating partner in CT for secondary school marine science education). On March 11, 2014, we sent approximately 500 grams of *Gracilaria vermiculophylla* from UCONN to UCSD-SIO for use as seedstock for their planned laboratory-scale spray culture experiments. UCONN is continuously cultivating *Gracilaria* strains in its laboratories to provide sufficient biomass for year 2 experiments at UCSD-SIO and NCM. During the year 2 project period, we are planning to make additional collections and isolations of *Gracilaria* and *Ulva* from Mexico (no cost to BARD project). The Mexico collections and isolations will, hopefully, allow us to establish new strains and species that potentially possess higher tolerance to desiccation, high solar irradiance, and high temperature; adaptations that will favor scale-up spray culture in greenhouses.

***Ulva*:** During the current project period, we isolated two new strains of *Ulva* from Bridgeport, CT and Jamaica Bay, Queens, NY. Healthy thalli were transported to the laboratory on ice within 24 h. Punched disks from marginal sections of the thalli were cleaned by gently wiping with sterile cotton balls. The disks were transferred to von Stosch's enriched seawater (VSE) in 50 mm sterile Petri dishes and maintained at 20°C, under 20 μmol m^{-2} s^{-1} artificial light, and day neutral photoperiod (12:12, L:D) (Ott, 1965). By the following day, spores or gametes were released from the disks, and a few spores were placed on slides (10mm X 10 mm) in sterile Petri dishes for the development of new thalli. We are currently working on species identification from these collections using tufA and ITS genes.

Additionally, *U. prolifera*, and *U. linza* (collected from Rudong Sea, China) were transferred from Prof. P. He's laboratory (Shanghai Ocean University, China) to UCONN's laboratory. The *U. prolifera* is the same haplotype of the world's largest macroalgal bloom forming strain from The Yellow Sea, China. Currently, UCONN is maintaining these four *Ulva* strains in its laboratory, cultivating biomass of these strains to provide sufficient biomass for year 2 experiments at UCSD-SIO and NCM (Fig. 1).

Figure 1. Clone cultures of *Gracilaria* (left) and *Ulva* (right) at UCONN's Stamford Laboratory.

New culture media development for *Gracilaria*[1]: The commonly used nutrient medium for red algal cultivation is the von Stosch Seawater enrichment (VSE) media (Ott, 1965). VSE contains several important nutrients including nitrate, phosphate, iron, manganese, EDTA and vitamins. However, VSE is not applicable for large scale cultivation due to its high material and preparation costs. To address this commercial-scale necessity - inexpensive media costs, we have evaluated two potential culture media for *Gracilaria* cultivation using commercially available fertilizers (Table 1).

Table 1. Nutrient composition in each media

	VSE	JS	MG
Nitrogen	500 μM	500 μM (41% NH_3 and 59% NO_3)	500 μM (15% NH_3 and 85% Urea)
Phosphorus	30 μM	39 μM	34 μM
Iron	1 μM	0.6 μM	0.8 μM
Manganese	10 μM	0.3 μM	0.3 μM
EDTA	10 μM	not specified by maker	not specified by maker
Vitamins	Yes	-	-

Since vitamins are included in VSE, five media conditions were utilized in a comparative growth study: 1) VSE, 2) JS, 3) JSV, 4) MG and 5) MGV. Total nitrogen and phosphorus concentrations in each medium were adjusted as same those in VSE (500 μM and 30-39 μM, respectively; Table 1). However, the nitrogen sources were different in each media (VSE, 100% NO_3, JS, 41% NH_3 and 59% NO_3 and MG, 15% NH_3 and 85% Urea). *Gracilaria* was

[1] This study was also supported by the U.S. EPA Long Island Sound Study's Long Island Sound Futures Fund, New York State Attorney General's Bronx River Watershed Initiative Grant Program, National Fish and Wildlife Foundation (NFWF/Legacy Grant Project IDs: 1401.10.024266 and 8012.08.030370) and Connecticut Sea Grant College Program (R/A-38).

cultivated at 100 μmol m^{-2} s^{-1} artificial light at 20°C, and 12:12 L:D photoperiod. The stocking density was 2 g/L. At one-week intervals, and for four weeks running, all of the biomass in each flask was weighed (fresh weight; FW) and samples were taken for tissue analyses for total N content. Tissue samples were dried at 60 °C before being ground, then the powder was analyzed using a Perkin Elmer 2400 series II CHNS/O elemental analyzer.

Gracilaria cultivated in the medium with JS fertilizer grew as well as the batch cultured in VSE. Growth rates in VSE, JS and JSV were 6.8-11.0%, 5.8-11.8% and 6.2-10.5% d^{-1}, respectively. Growth rates using MG (5.5-8.1% d^{-1}) and MGV (4.5-7.4% d^{-1}) were lower than those in other conditions (Fig. 2). Tissue nitrogen contents were also higher at VSE, JS and JSV than at MG and MGV (Fig. 3). Vitamins did not show any significant effect on the growth of this species, at least over the 7 days growth between culture medium change (Fig. 2, 2). When the price of each media was compared (Table 2), JS proved to be the least expensive ($0.01 per m^3 of medium) compared to VSE at $1.62 per m^3. Although the costs for vitamins were removed from VSE, the cost for CF1 media is still 2% of the cost for VSE without vitamins. This result suggests a potential uses of commercially available fertilizer in seaweed nursery systems.

Table 2. Price comparisons of different culture media

Nutrients	Price per m^3 of culture medium
VSE	$1.623
JS	$0.010
JSV	$1.114
MG	$0.012
MGV	$1.116

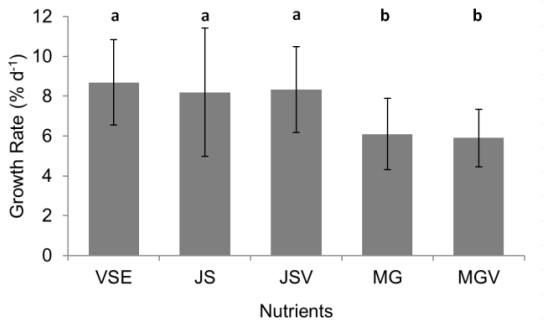

Figure 2. Comparison of growth rates of *Gracilaria tikvahiae* grown at different fertilizers. VSE: von Stosch enrichment media; JS: Jack's Special™ only (JS; N:P:K, 21:8:18); JSV: JS with vitamins; MG: Miracle-Gro® only (MG; N:P:K, 24:8:16); MGV: MG with vitamins.

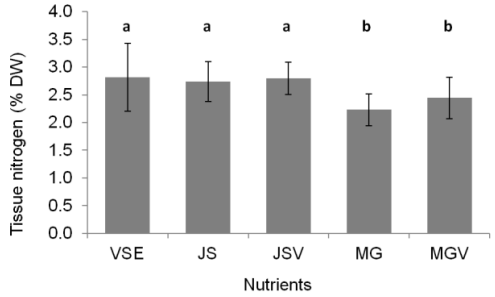

Figure 3. Comparison of tissue nitrogen (N) of *Gracilaria tikvahiae* grown at different fertilizers. VSE: von Stosch enrichment media; JS: Jack's Special™ only (JS; N:P:K, 21:8:18); JSV: JS with vitamins; MG: Miracle-Gro® only (MG; N:P:K, 24:8:16); MGV: MG with vitamins

Characterization of the growth capability of the world largest bloom forming species, *Ulva prolifera* [2] : *Ulva prolifera* has been identified as the dominant species in the world largest macroalgal blooms in the Yellow Sea of China. Some *Ulva* species are known to have polarized growth when the thalli were cut into small pieces. Miiller-Stoll (1952) first observed that in *U. compressa*, new rhizoids were formed at the basal cut of the thalli while the upper cut surfaces showed the non-rhizoidal cells, called 'papillae'. Eaton et al. (1966) and Moss & Marsland (1976) also found a similar phenomenon in *U. intestinalis*. The objectives of this study were to identify the different developmental strategies in *U. prolifera*, to determine environmental conditions forming the polarized growth.

Fresh green tubular thalli without branches were selected and cut into 5 mm segments in all experiments. The segments were transferred into Pyrex Petri dishes (7 cm in diameter) containing 5 mL VSE medium. The experiments were performed at irradiances of 50, 75 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperatures from 10 to 25°C in 5°C for two weeks. We found that one 5 mm segment of *U. prolifera* could form 25 new thalli on average, and each thallus grew matured releasing spores in four weeks (Fig. 4). Among *Ulva* species, this growth strategy was reported only in *U. prolifera.* Previous studies also showed the polarized growth patterns in *Ulva* species, however, other *Ulva* species including *U. compressa*, formed only one or two new thallus from the upper cut surfaces. Another interesting finding in the present study is that the polarized growth was mostly observed at high temperature (≥ 20 °C) and low light (≤ 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions. Otherwise, most segments completely released germ cells. This growth and reproductive patterns in *U. prolifera* segments can explain the outstanding growth of this alga, and forming the world largest macroalgal blooms in China.

Publications:

Kim J.K. and Yarish C. 2014. Development of a sustainable land-based *Gracilaria* cultivation system. Algae (Journal); Accepted on Sept 5th, 2014. Expected publication in October, 2014.

[2.] This study was also supported by a grant to P. He and J. Zhang from the Ocean Public Welfare Scientific Research Project, China (201205010).

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2) Partner Organization: UCSD, Scripps Institution of Oceanography (SIO)

Task / Objectives:

Descriptive Title of Work: Laboratory Growth performance of *Gracilaria vermiculophylla* for three different re-circulating seawater spray system designs.

Introduction: The overall project aims to develop an economical land-based (within greenhouses) commercial-scale, spray culture system for the cultivation of high protein *Gracilaria* (red seaweed) and Ulva (green seaweed) biomass to provide a sustainable source for augmenting protein content in formulated marine shrimp diets. The on-land, spray cultured source would serve as a sustainable alternative to ocean cultivation for agar, or biomass for renewable energy.

Methods: During the first year, SIO developed and tested three different structural cultivation designs for growing *Gracilaria vermiculophylla* (Strain ID: G-NY-ST6) (Gv) in a laboratory-scale recirculating seawater spray system in laboratory scale. The three systems were: plastic / bag-string vertical design (BSVD); multi-level horizontal (MLHD); and netted vertical design (NVD). Growth rates and protein production of seaweed using these cultivation methods were compared with submerged Gv cultures. The interaction effect of cultivation designs used a four-level single factorial randomized block experimental framework ($p<0.05$). In another test, the best performing cultivation design used the four-level two factor experimental design to test interaction effects of light and $NaHCO_3$ addition for growth performance and protein production content of Gv. Pre-tests of seaweed growth used in-bottle and 6-well experiments

to determine the range of light and NaHCO3 conditions for seaweed growth in the culture design-units. Stocking density, media, biomass to volume ratio were accounted also as parameter inputs for the experimental design spray system. A mini-spray culture experimental array, in a randomized framework (Figure 1), was employed to perform testing on the UCONN-isolated strains of Gv for physiological tolerance to culture protocol factors of light and $NaHCO_3$ using von Stoch media (renewed twice weekly). Three replicate 1 L polycarbonate culture bottles, 6-well plates and polyethylene containers were used per test condition.

Growth rate (as %GR/day) was calculated using the difference of the final and initial weight divided by the number of days in the culture and expressed as % [(Wt/Wo)1/t-1*100] (Yong et al., 2013). The relative growth rate per day (RGR/day) was computed using the formula (ln Wt-lnWo/t) * 100. (Glen and Doty 1992). Each experimental design-unit contain twelve pieces of ~1 g of pre-weighed seaweed seedlings (total trial = 12). The high number of trials in each replicate unit minimizes the effect of lost branches during seedling preparation and during harvest. Three replicates for each design cultivation unit were used, incorporating three spray system designs and one submerged system design: Multi-level horizontal design (MHLD), (Bag-String Vertical design (BSVD), Netted-Vertical Design (NVD) and S(submerged System) : See photo above for graphical representation of each design. The protein content in the seaweed was extracted using an optimized method detailed in (Lopez et al., 2010). Protein concentrations in sample extracts were analyzed based on the absorbance (@ 750 nm) against the BSA reference standard (Lowry et al., 1951). Normality of data were tested using Shapiro-Wilkinson test and Brown-Forsythe for variance equality test. The effects of the culture conditions on the growth rate and protein content was verified through one-way and two-way ANOVA. This was followed by Holm-Sidak method (significance level of 0.05) for the pairwise multiple comparison.

Results: The 6-well plate platform showed HL as high GR. The difference in the mean % GR/day values among the different levels of light is greater than would be expected by chance after allowing for effects of differences in the $NaHCO_3$ levels. There is a statistically significant difference ($P = <0.001$). To isolate which group(s) differ from the others, a multiple comparison procedure employed showed highest GR/day at high (HL) condition with significant variation with GR/day at medium light condition. The $NaHCO_3$ addition and

its interaction (two-way anova) light as a factor did not show any significant effect on the %GR/day of Gv suggesting that light is controlling factor that led to the increase of the seaweed's growth rate and not the added $NaHCO_3$.

Even at higher stocking density in a glass bottle experiment , addition of NaHCO3 did not result to any significant growth differences among growth rates of Gv grown in difference $NaHCO_3$ levels (one-way ANOVA; P = <0.05). Comparing the with the 6-well plate experiment, the %GR/day is relatively lower. This is due to the lower light level in the bottle experiment. However, the gain weight of Gv of the glass bottle experiment is relatively higher in grams gain than in the 6-well plate. This suggests that the initial biomass weight of the seedling and material used in the culture are both factors that would affect %GR/day and the relative growth rate.

MLHD provided a significant growth increase in comparison with the other cultivation design units. The NVD showed the lowest growth rate. The NVD growth rate can be attributed to the proximity of the netted panels, which caused shading to some of the seaweed experimental units. Addition of $NaHCO_3$ and the kelp extract resulted in a significant decrease in the growth rate of Gv, and a significant depression in protein content in Gv biomass. This result suggests that the addition of $NaHCO_3$ and kelp extract are not desirable ingredients in the growth media for Gv.

It is projected that using a glass material in a MLHD configuration at the high light condition with the same indoor lab-scale system, would result in a relative growth of 21 (g/g/m2/day; Table 2). In an outdoor cultivation, using a more transparent culture array, could potentially result in a relative growth for Gv as high as 433 g/g/m2/day, with an estimated protein production of 48 g/m2/day.

The Multi-level Horizontal design (MLHD) provided the highest growth rate of Gv in a recirculating system. However, we concluded that the best growth rate could potentially be obtained using a more transparent, glass type of material rather than using PE plastic containers. Based on these experimental results we project that improved growth rates and protein content should be obtainable under outdoor, higher solar light conditions as would be found in a glass-glazed greenhouse. We will further explore other input parameters (e.g.,

nutrients and CO_2) to maximize growth and protein production of Gv in spray recirculating system.

Figure 1. A randomized two factorial experiment that determined the effect of cultivation design on growth of *Gracilaria* at high light condition with and without addition of NaHCO3 + kelp extract (a potential growth enhancer). The four type of designs were" (abbreviation, see text) MLHD, BSVD, NVD and S. Light level was 141.0±5.5µE/m^2/s; pH: 8.94±0.22; T =28.5±0.99.

Publication / Presentation:

Wilson G. Mendoza, Dominick Mendola, Patricia Abelin, and B. Greg Mitchell. Growth performance of *Gracilaria vermiculophylla* for three different re-circulating seawater spray system designs. Poster presented at the Algae Biomass Organization's Summit meeting in San Diego, CA Sept. 29 to Oct 2, 2015.

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3) Partner Organization: Israel Oceanographic and Limnological Research, National Center for Mariculture (IOLR-NCM)

Task / Objective: Objective 3. Cultivate several species in kg quantities (dw) for use in experimental diets for marine shrimp.

Introduction: In year 1, two separate spray-culture efforts progressed NCM towards the overall goals of the project:

1. An experimental outdoor spray irrigation system was designed, built and experimentation begun by Mr. Yossi Bronfman, who studying for an MSc degree in

the Hebrew University of Jerusalem. The site for these experiments is on the coast of the Mediterranean Sea, hosted by the Mariculture Center in the Mevoot-Yam School (Fig. 1).

The units are fed by surface water from the adjoining Mediterranean Sea through a pump, a holding tank and a second pump. The water sprays on to the top of each tray at 100L/hr., and drains from the trays into a 210-L drainage tank, from where it is pumped back up to the influent header. The drainage tank water is replenished continuously with clean seawater about twice/day. The algae collected at the end of each trial from each tray is observed for its appearance, epiphytes and additional parameters, then rinsed, dried in a 60^{o}C oven for 2 d, and collected individually in a bag in a desk desiccator until shipment to TAMU. Samples are taken for nutrients, and records are kept of temperature, pH, sunlight.

2. The second system is a 9-unit (1m^2 each) spray-culture array aimed to grow kilograms of macroalgae for the shrimp feed diets. The array has been designed, built and operated at the National Center for Mariculture (NCM) in Eilat, Israel (Figs 2, 3). The units are spray-fed using fishpond (grey mullet) effluents from an adjoining sump pond. Water is sprayed to the top of each tray at about 40 L/ h. Each tank is loaded with 1.5 kg of live, drained *Ulva* biomass. After growing for 7-10 days, each tank is harvested and restocked with 1.5 kg of drained *Ulva*. The added growth increment for that period is removed, rinsed and shade-dried for several days. The incrementally-harvested and dried biomass is crushed and put in a bag for eventual shipment to TAMU. Water samples are regularly taken for nutrient analysis.

3. The first shipment of dried samples grown in the 9-unit outdoor array was shipped from NCM to TAMU in August, 2014. These samples will be used by YAMU for proximal analysis and for consideration in experimental shrimp diets.

4. Toward the building of a greenhouse, a design has been made, the plastic cover has been purchased, and an installing contractor has been contracted after a bidding procedure. The greenhouse should be completed this fall.

5. A commercial "spinner: unit (for de-watering biomass) is in the process of procurement. This unit will allow us to completely de-water large batches of algal biomass grown and harvested from the greenhouse.

6. Once the greenhouse is completed, the culture units may be moved into the greenhouse, for the winter season.

7. We anticipate, that sometime in the early months of the 2nd year, NCM should be ready for the 2014-2015 winter season spray-culture trials. For those trials, we anticipate receiving a shipment of laboratory-cultured macroalgae from project partner UCONN.

Figure 1: An experimental spray-irrigation system in Mevoot Yam. Each of the 6 trays has a culture area of 0.12 m^2. Note the water spray at the top of each tray, with drainage at the bottom. Three of the trays are placed in a horizontal orientation, the other trays are slanted at a 6^o angle.

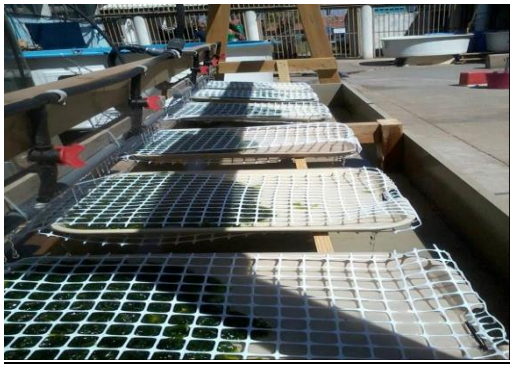

Figure 2: The large scale spray-irrigation culture system in Eilat, during construction. Each of nine units has an area of 1 m^2

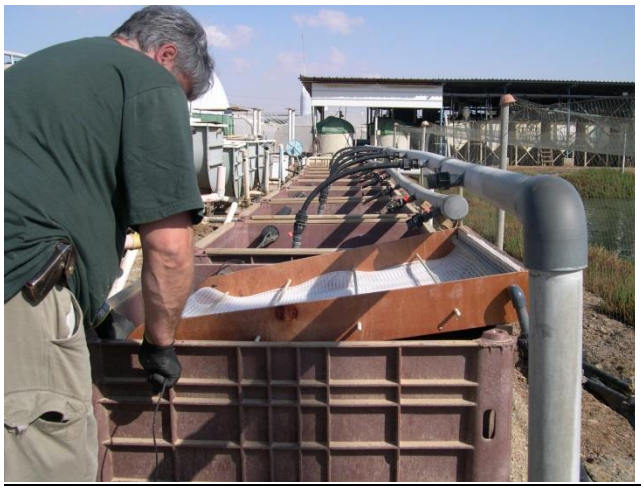

Figure 3 (right): The Eilat spray-irrigation culture system with a ready-to-harvest crop of locally-grown *Ulva lactuca. The sump pond is in the background.*

In Yr-2, NCM will test the best performing strains of *Gracilaria* and *Ulva* sent from project partner UCONN, plus a strain selected locally in Israel. Each strain will be grown out of doors in laboratory-scale spray culture trays, and also in scaled-up spray-culture in a greenhouse (to be completed in early Yr-2). Strains will be tested against each other for overall growth and biomass production, protein content and lipid spectrum.

Seawater and nutrients for the spray-culture growth trials will be supplied from near-by commercial fish ponds and pumped to the spray-culture greenhouses. Use of strains from the USA is justified, since it can be expected that deployment of the developed technology will begin in the USA, and will be contained in land-based cultivation units.

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4) Partner Organization: Texas A & M University, AgriLife Research (TAMU)

Tasks / Objectives: 2, 4 & 5
Descriptive Title: The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aqua-feeds

Objective 2. Formulate defined and enriched seawater-based growth media for macroalgae designed to increase cellular protein content (UCONN). **Task 2.1.** This task did not require any action taken by AgriLife Research. **Task 2.2.** AgriLife Research (at TAMUS) is scheduled to analyze protein/amino acid content of lab-supplied seaweed samples from NCM, while lipid content of the samples is scheduled to be analyzed at UCSD-SIO.

Results from preliminary testing performed before the approval of funding for the current project showed that protein content *Gracilaria* and *Ulva* can be modified by culture conditions (see table which follows).

Macroalgae Type	Crude Protein (%)	Crude Lipid (%)	Ash (%)	Moisture (%)
LIS Outdoor *Gracilaria*	34.6	-	28.0	1.5
"Low Protein" *Ulva*	41.0	1.3	13.4	12.3
"High Protein" *Ulva*	40.1	1.3	12.2	10.1

Samples of macroalgae, cultured under different conditions at the NCM, are currently being transferred to AgriLife Research. As protein level is the most significant component in diet formulations, all samples will be first analyzed for protein content. Samples with the highest protein levels will be further tested for moisture, fiber, crude lipid, ash, energy, calcium, phosphorus and amino acid with emphasis on lysine and methionine. These samples will also be sent to UCSD-SIO for more extensive lipid content analyses. As stated in the objectives, the information to be collected from these analyses will be entered into a linear least-cost formulation software to prepare the test diets.

Objective 4. Design and prepare experimental seaweed-based pelleted shrimp diets based on the composition of commercial shrimp diets and the composition of the produced macroalgal meals (AgriLife Research);

Task 4.1. To determine the ability of macroalgal meal to replace the alginate binder, test diets will be formulated with seaweed meals (incorporated at expected fishmeal replacement levels) and 2, 1 or 0% alginate binder. Feed water-pellet stability will be measured.

Task 4.2. To ensure diets are optimally formulated, protein and energy digestibility will be determined for the protein enriched seaweed meals prior to dietary formulation. These studies are scheduled to start only in the second year.

Objective 5. Conduct shrimp feeding trials with the experimental macroalgae diets in comparison with a commercial aquaculture shrimp aqua-feeds (AgriLife Research). These studies are scheduled to start only in the second year.

Description of the Cooperation:

During the first year of performance on the project, Drs. Mitchell & Mendola at UCSD-SIO have maintained close e-mail communication with all BARD project partners, and regular telephone contact with Professors Yarish & Jang (UCONN), and Prof. Samocha at TAMU. Close coordination has been maintained between UCONN & UCSD-SIO to facilitate transfer of Gracilaria spp. algal biomass from UCONN to SIO required for SIO's lab-scale spray culture experiments herein reported.

In Israel, Dr. Neori's work has been somewhat delayed due to the unrest situation in the country, and the real and daily dangers of working out of doors at the National Center for Mariculture in Eilat, and elsewhere in the country. Despite these near daily assaults, Dr. Neori managed to guide the construction of his experimental array for spray culture experiments at NCM, and complete a number of the planned yr-1 experiments. During these trying times, e-mail communication was maintained between each of the US-partners and Dr. Neori, both for moral support of he and his family, and for technical discussions on the design and implementation of his system and experiments. All-in-all the communications were very well maintained and useful to progress the aims and tasks of the project.

In early October, 2014, Prof. Tzachi Samocha (TAMU) traveled to Israel to visit with Dr. Amir Neori (NCM) to discuss the initial results from Dr. Neori's experiments. Prof. Yarish (UCONN) talked with Prof. Samocha in anticipation of his trip to Israel, to suggest that he convey to Dr. Neori that he should consider increasing the nitrogen content of his spray culture media using either a commercial fertilizer mix, or high-nitrogen fish farm effluent. Dr Yarish's suggestion was based upon encouraging results from recently published work from UCONN on growth increases witnessed in larger-scale, submerged growth trials with Ulva and Gracilaria conducted at UCONN and reported in the recent publication submitted by UCONN (see below).

Communications are continuing into the 2nd year of the project using both e-mail and (within the USA consortium..) telephone calls and teleconferences. On occasion, telephone calls are made between one or more of the USA partners and Dr. Neori in Israel.

Changes in the direction of the project:

NO substantive changes from the original work plan and task list were requested during the first year of performance.

Publications & Presentations:

Kim J.K. and Yarish C. 2014. Development of a sustainable land-based *Gracilaria* cultivation system. Algae (Journal); Accepted on Sept 5th, 2014. Expected publication in October, 2014.

Wilson G. Mendoza, Dominick Mendola, Patricia Abelin, and B. Greg Mitchell. Growth performance of *Gracilaria vermiculophylla* for three different re-circulating seawater spray system designs. Poster presented at the Algae Biomass Organization's Summit meeting in San Diego, CA Sept. 29 to Oct 2, 2014.

END - First Annual Scientific Report

YEAR 2 - Research Results

1) Partner Organization: University of Connecticut (UCONN)

Objective 1. Collect and Screen red and green seaweed candidates from several genera including *Gracilaria* and *Ulva* for temperature tolerance and growth under spray cultivation conditions.

Strain Isolation:

During year 1, we isolated two new strains of *Ulva* from Bridgeport, CT, and Jamaica Bay, Queens, NY. During the current project period (Oct. 1, 2014 – Sept. 31. 2015), the species were identified as *U. compressa* and *U. laetevirens*, respectively, using tufA and ITS genes. Additional strains of *Ulva* and *Gracilaria* were isolated using samples collected by Dr. Charles Yarish during his sampling trip in Mexico (Oct. 18, 2014 – Oct. 19, 2014). The *Ulva* was originally collected from the Melcone Beach (the main beach) of La Paz, Baja California Sur, MX. It was initially identified as *U. ohnoi*. The *Gracilaria* was collected from Pichilingue, La Concha and Las Pacas, Baja California Sur, Mexico. These new strains were isolated at UCONN. The *Gracilaria* species was identified as *G. parvispora* by DNA

sequencing using ribosomal small-subunit 18s RNA. This species is an invasive species to Mexico (native to Hawaii, USA). All these strains of *Ulva* (Strain ID: U-MX-ST1) and *Gracilaria* (Strain ID: G-MX2-ST1) are currently being maintained at UCONN.

Material Transfer from UCONN to BARD partner institutions:

UCONN has continuously increased biomass of *Ulva compressa* and *Gracilaria vermiculophylla* at our UCONN and Bridgeport Regional Aquaculture Science, Technology Education Center (BRASTEC) labs in volumes ranging from 1-200L (Figure 1). The biomass has been made available for experimentation at UCONN, as well as in partner institutions. On Dec. 8th 2014, UCONN sent approximately 500 grams, fresh weight of *Ulva compressa* (strain ID: U-CC-ST1) to UCSD-SIO for experiments. Additional 250 grams, fresh weight of *Ulva compressa* (strain ID: U-CC-ST1) was sent to UCSD-SIO on Aug. 24th 2015.

During Dr. Yarish's UCONN sponsored trip to Israel (Jan. 7, - Jan. 9, 2015), Dr. Yarish delivered UCONN's *Ulva compressa* (U-CC-ST1) and *Gracilaria* (G-NY-ST4 and GV-KR-ST1) strains to Israel Oceanographic and Limnological Research Institute and The National Center for Mariculture (Haifa and Eilat).

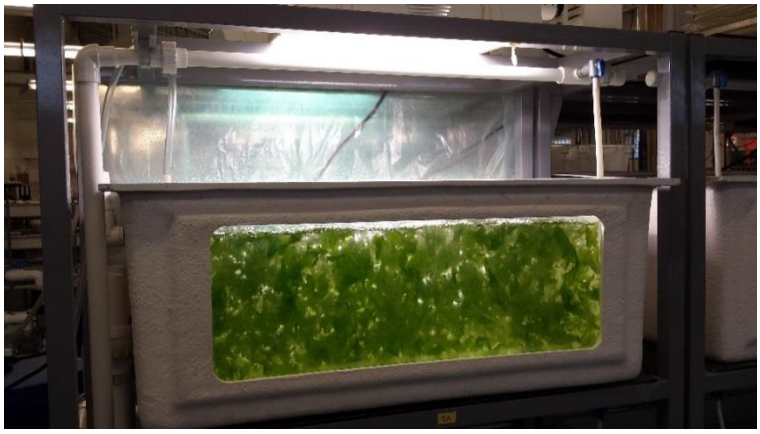

Figure 1. *Ulva compressa* growing in 200 L tank at Bridgeport Regional Aquaculture Science and Technology Education Center (BRASTEC)

1-1. The effect of temperature on growth and tissue composition of the non-native seaweed *Gracilaria vermiculophylla* in the Long Island Sound compared to native *Gracilaria tikvahiae*[2]

[2] This study was also supported by the U.S. EPA Long Island Sound Study's Long Island Sound Futures Fund, New York State Attorney General's Bronx River Watershed Initiative Grant Program,

In the past two decades, the red alga *Gracilaria vermiculophylla*, a species native to the waters of Korea and Japan, has invaded marine coastal areas of Europe and the Americas, thriving in conditions that differ from those of its native habitat. In recent years, *G. vermiculophylla* has been discovered in the Long Island Sound (LIS) growing alongside the native congener *Gracilaria tikvahiae*. The goal of this study was to determine whether the *G. vermiculophylla* strain growing in LIS exhibits phenotypic plasticity, and whether physiological differences can explain the success of the invasive species. Two strains of *Gracilaria vermiculophylla* (isolated in Korea, GV-KR-ST1 (courtesy of S.M. Boo, Daejeon, Korea) and LIS, G-NY-ST4) and a strain of LIS native *Gracilaria tikvahiae* (G-RI-G1) were grown in von Stosch's enriched (VSE) medium for four weeks under temperatures ranging from 20° C to 34° C using a temperature gradient table, while all other environmental conditions (100 µmol m^{-2} s^{-1}, 12:12 L:D photoperiod and 30 ppt salinity) remained constant. At the end of each week, the wet weight of each sample was recorded. Thalli were reduced to the original stocking density of 1 g L^{-1}, excess biomass was preserved for tissue carbon and nitrogen analysis, and water samples were collected.

Growth Rate – *G. vermiculophylla*

Overall, the LIS strain of *Gracilaria vermiculophylla* appeared to grow at rates more similar to *G. tikvahiae* than to those of the Korean strain. At each of the tested temperatures, the Korean strain of *G. vermiculophylla* outperformed the LIS strain and *G. tikvahiae*. During the first week, at 24 °C and 29 °C the Korean strain of *G. vermiculophylla* grew at rates of 22.9% and 23.2%, respectively. By week four at 24 °C and 29 °C, the Korean strain grew at only at 10.0% and 9.4%, respectively. Under the same conditions in the first week the LIS strain grew at 6.2% and 5.7%, and *G. tikvahiae* grew at 12.0% and 9.4% (Figure 3, 4). For 20, 24, and 29 °C treatments, all strains displayed a general decline in growth rate over time (Figure 2-4). However, at 34 °C, the growth rate of both *G. vermiculophylla* strains slightly increased. *Gracilaria tikvahiae* failed to survive at 34 °C bleached within the first week of exposure and was removed from growth experimentation. By the end of the second week, one replicate of *G. tikvahiae* at 29 °C failed to survive and it too was removed from experimentation (Figure 5).

National Fish and Wildlife Foundation (NFWF/Legacy Grant Project IDs: 1401.10.024266 and 8012.08.030370) and Connecticut Sea Grant College Program (R/A-38).

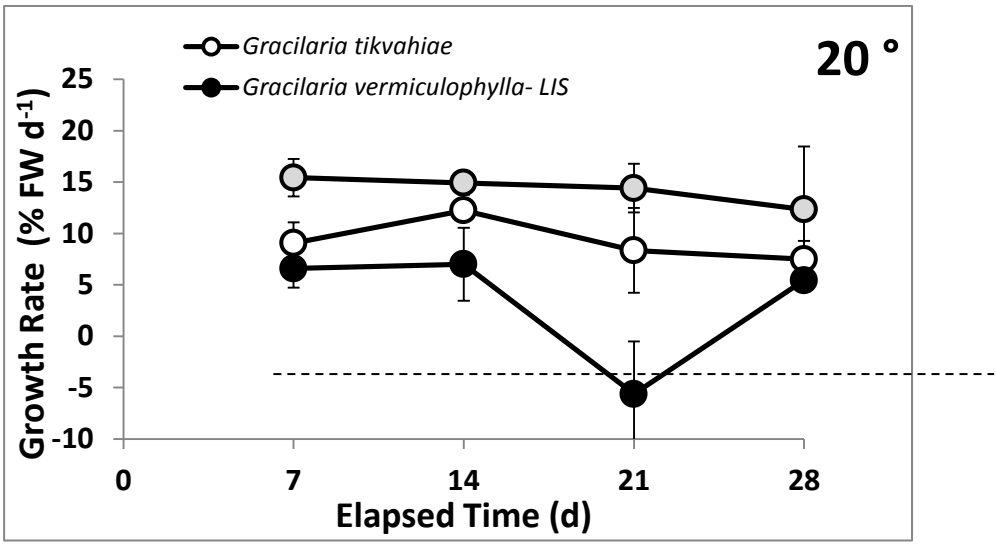

Figure 2. Average weekly growth rate measure in percent growth per day of each *Gracilaria* strain under the 20 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

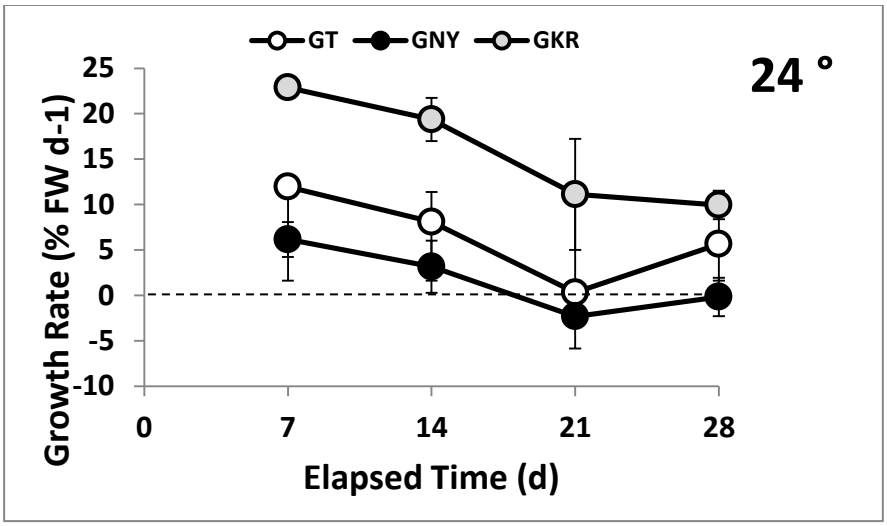

Figure 3. Average weekly growth rate measure in percent growth per day of each of *Gracilaria* strain under the 24 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

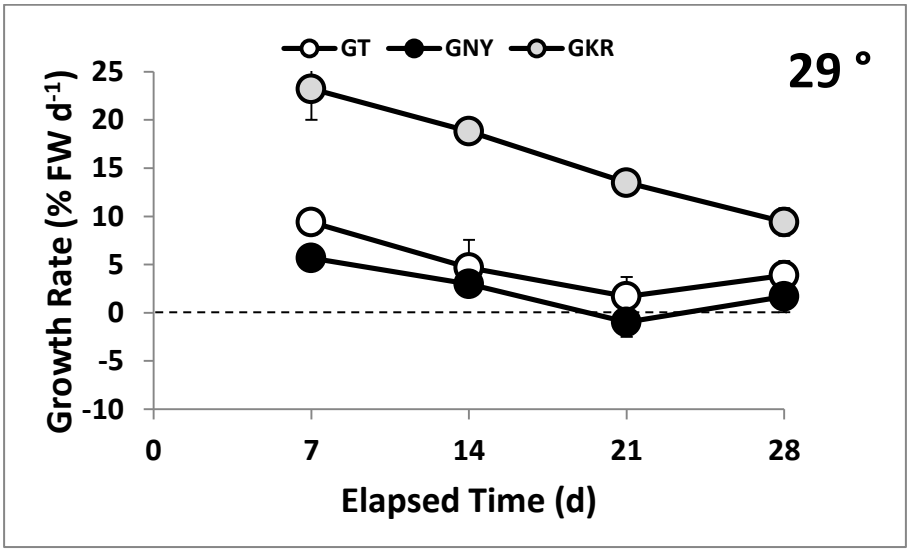

Figure 4. Average weekly growth rate measure in percent growth per day of each *Gracilaria* strain under the 29 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

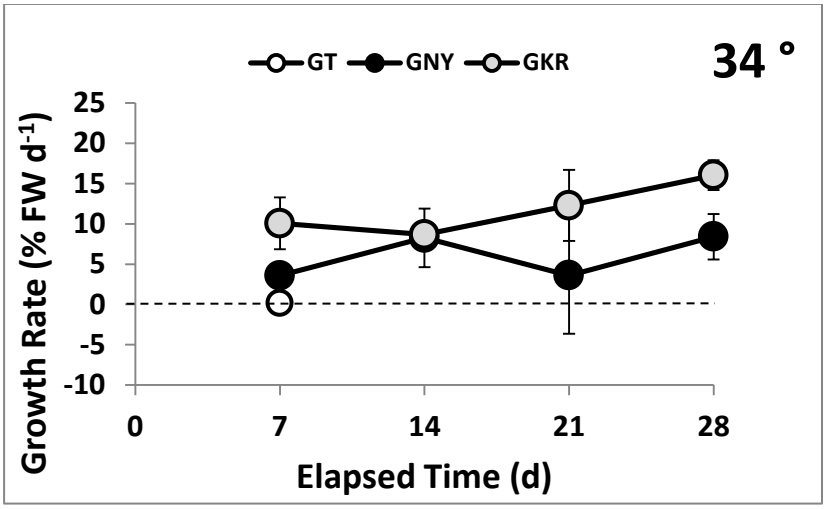

Figure 5. Average weekly growth rate measure in percent growth per day of each *Gracilaria* strain under the 34 °C temperature treatment. Error bars are not present for plots with minor standard deviation. *Gracilaria tikvahiae* is not displayed past week one as no replicates survived under this condition.

Tissue Nitrogen - *G. vermiculophylla*

For each temperature treatment, the LIS strain of *Gracilaria vermiculophylla* consistently concentrated more N in its tissue than the Korean strain and the LIS native *G. tikvahiae*. There was one exception at 34 °C treatment in week 4 where the Korean strain had a higher average concentration (Figure 9). The LIS strain exhibited nitrogen levels ca. 4-5% N (DW), whereas the Korean strain and *G. tikvahiae* produced tissue with 2-3% N, DW (Figure 6-9).

For all three strains, tissue N remained relatively constant over the four week period. For both the LIS and Korean strains of *Gracilaria vermiculophylla* tissue N appears to be influenced by temperature; as temperature increased, tissue N tended to decrease. However, this trend was not apparent with *Gracilaria tikvahiae*.

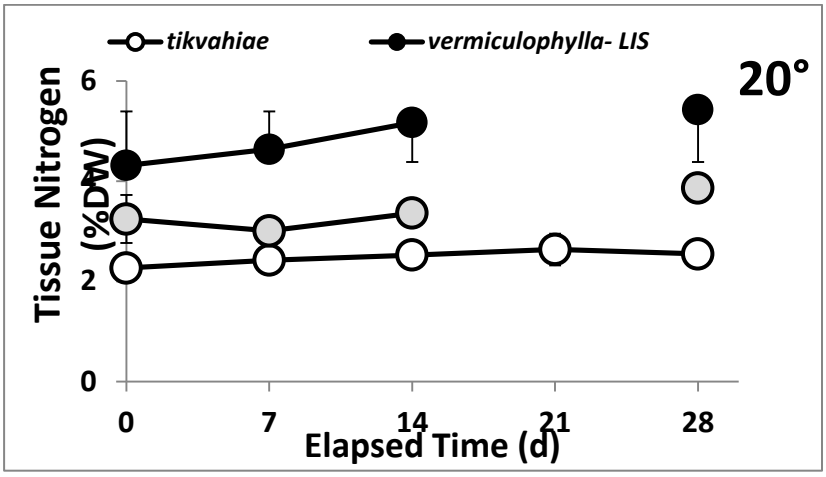

Figure 6. Average percent tissue nitrogen in each dried sample after each of four weeks for each *Gracilaria* strain under the 20 °C temperature treatment. The week three measurements for *G. vermiculophylla* strains were not made. Error bars are not present for plots with minor standard deviation.

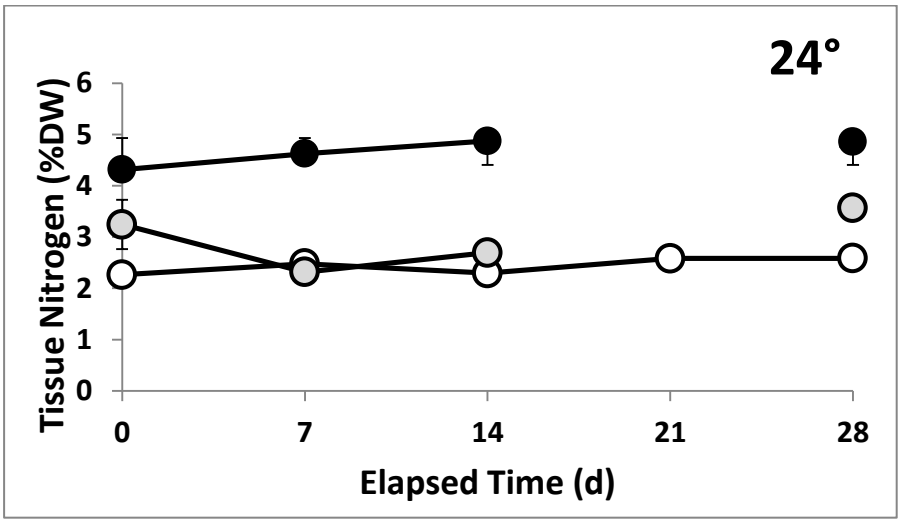

Figure 7. Average percent tissue nitrogen within each dried sample after each of four weeks for each *Gracilaria* strain under the 24 °C temperature treatment. The week three measurements for *G. vermiculophylla* strains were not made. Error bars are not present for plots with minor standard deviation.

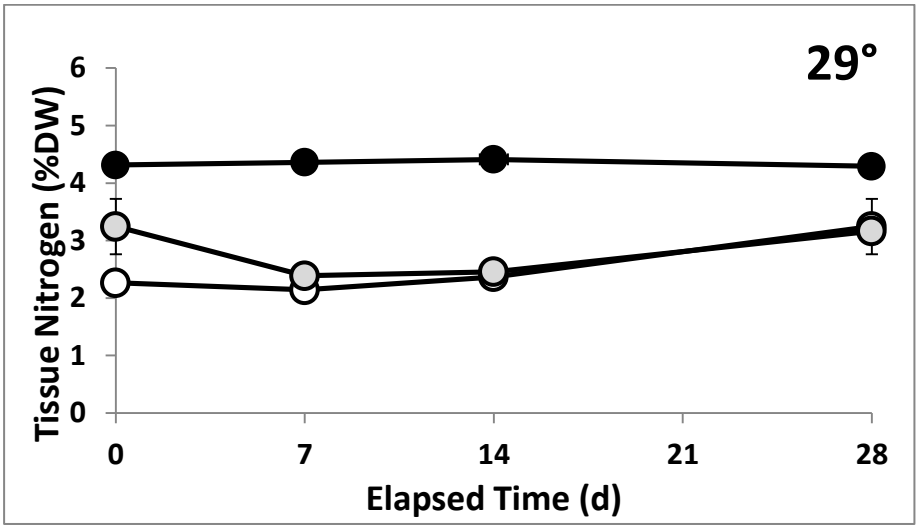

Figure 8. Average percent tissue nitrogen within each dried sample after each of four weeks for each *Gracilaria* strain under the 29 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

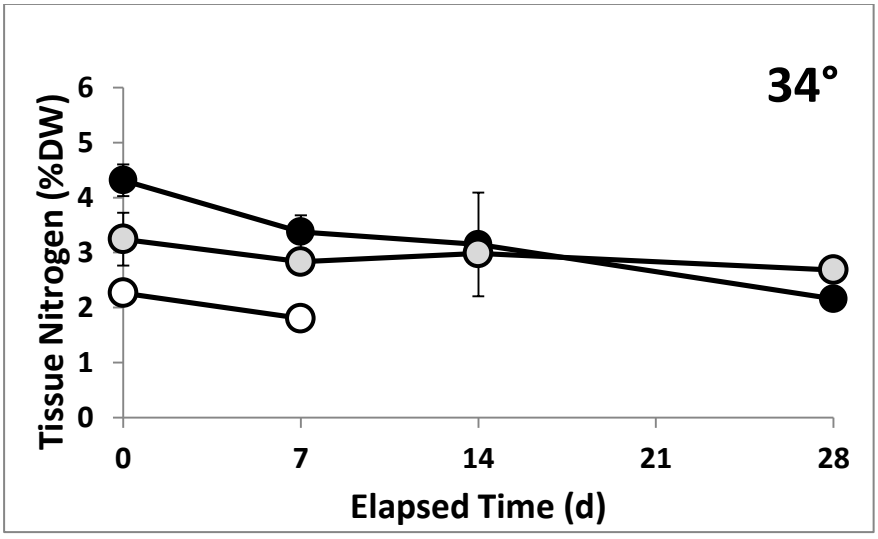

Figure 9. Average percent tissue nitrogen within each dried sample after each of four weeks for each *Gracilaria* strain under the 34 °C temperature treatment. *Gracilaria tikvahiae* is not displayed after week 1 as no replicates survived under this condition and was therefore removed from the study.

Statistical Evaluation of Results An ANOVA was conducted on results of growth rate and tissue nitrogen for week four of the growth period and showed significant differences among strains for each temperature treatment with the exception of growth rate at the 20 °C treatment. For growth rate, the *Gracilaria vermiculophylla* strain from Korea always had faster growth rates than rates of the other strains at 24, 29, and 34 °C temperature treatments. The difference between the growth rates of *G. vermiculophylla* LIS strain and *G. tikvahiae* at 29 °C was not significant

(Figure 10). The difference between tissue N concentrations of each strain was significant for every temperature with the exception of the 29 °C treatment. In the 29 °C during week 4, no significant difference between the Korean strain of *G. vermiculophylla* and *G. tikvahiae* were found (Figure 11).

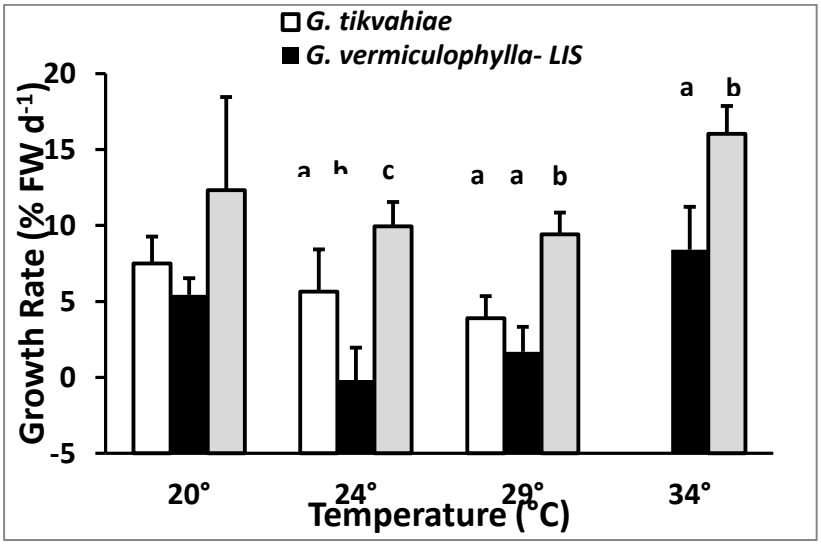

Figure 10. Average growth rate during week 4 in percent growth per day for each *Gracilaria* strain under temperature conditions 20, 24, 29, and 34 °C. Letters above bars denote results of a Fisher LSD Test comparing the strains within each temperature treatment after an ANOVA revealed significant difference among strains for 24, 29, and 34 °C temperature treatments.

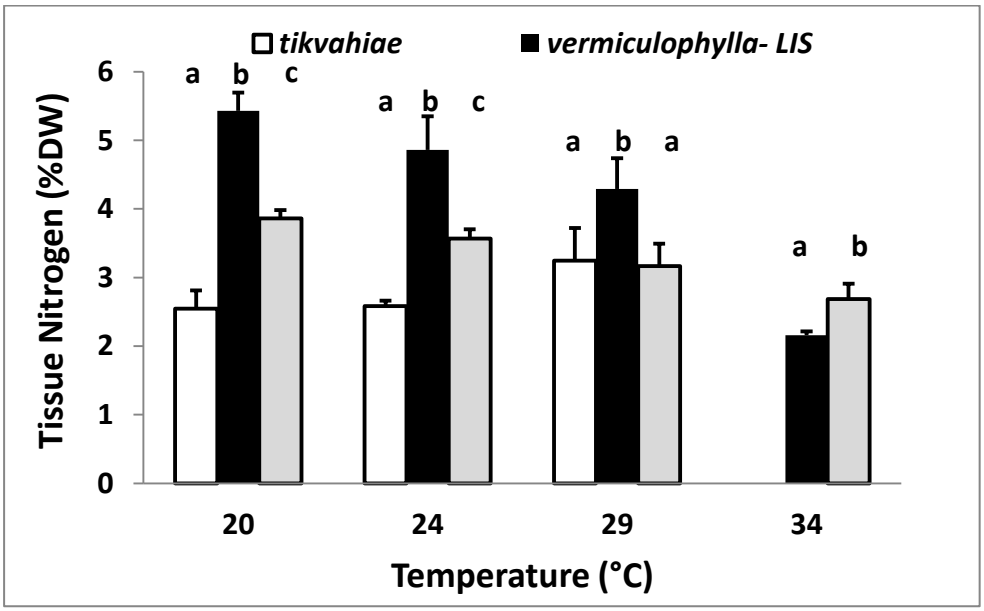

Figure 11. Average percent tissue nitrogen during week 4 for each *Gracilaria* strain under temperature conditions 20, 24, 29, and 34 °C. Letters above bars denote results of a Fisher LSD Test comparing the strains within each temperature treatment after an ANOVA revealed significant difference among strains.

1.2. Effects of desiccation in *Gracilaria* , simulating the spray cultivation systems, treated with *Ascophyllum nodosum* extract (AMPEP)[3]

The brown fucoid algae, *Ascophyllum nodosum,* commonly known as knotted wrack or rockweed, exist in the eulittoral zone. It is one of the most researched seaweeds in the northern hemisphere (Khan et al. 2010). An extract of *Ascophyllum nodosum* (Acadian marine plant extract powder – AMPEP) has been the basis of numerous studies, most recently with the economically important red macroalga, *Kappaphycus alvarezii* (Loureiro et al. 2010, Hurtado et al. 2009). *A. nodosum* extract (AMPEP) has proven to increase the temperature tolerance, otherwise considered lethal, as compared to control samples (Loureiro et al. 2010). AMPEP has also been shown to reduce epiphytes and increase disease resistance (e.g. "ice-ice" and "goosebumps") at concentrations of just 15 and 20 grams L^{-1} (Loureiro et al. 2010). Effects on terrestrial organisms have proven similar results. Cucumber plants exposed to 1% *Ascophyllum* derived solution applied twice daily for 10 days increased its resistance to a wide range of pathogens and parasites, while displaying increased biomass over the control samples (Jayaraman et al. 2010). The objective of this study was to determine if *A. nodosum* extract (AMPEP) increases the growth capacity of *Gracilaria vermiculophylla* and increases its tolerance to desiccation stress. Experimentation utilized a tide-simulating apparatus (Kim and Yarish 2010). This device allows macroalgae cultures to endure periods of submergence and exposure in regular intervals simulating tidal exposure. The flexibility in adjusting tide times for the tide-simulating apparatus (1 of table for treatment and another for a control with each table containing 18 individual replicate cylinders), gives an opportunity to explore emersion stress at regulated intervals, as well as static conditions (control or no emersion).

Gracilaria vermiculophylla (GV-KR-ST1) was cultivated in 2.5 L cylinders containing six different concentrations of AMPEP (0, 10, 20, 30, 40 and 50 mg /L; n=3) using the tide simulating apparatus. Of the 36 cylinders in total, 18 cylinders of *G. vermiculophylla* remained submerged while the *Gracilaria* in the other 18 experienced emersion stress with a 15 min / 1 min emersion/submergence cycle. A fifteen minute exposure caused approximately 10% water loss in *Gracilaria*.

[3] This study was also supported by the U.S. EPA Long Island Sound Study's Long Island Sound Futures Fund, New York State Attorney General's Bronx River Watershed Initiative Grant Program, National Fish and Wildlife Foundation (NFWF/Legacy Grant Project IDs: 1401.10.024266 and 8012.08.030370) and Connecticut Sea Grant College Program (R/A-38).

Emersion stress significantly affected the growth of *Gracilaria vermiculophylla*. When *G. vermiculophylla* didn't experience emersion stress, interestingly, addition of AMPEP reduced the growth rate of this alga. However, when *G. vermiculophylla* was exposed to air on a regular basis, a low concentration of AMPEP addition (10 mg L^{-1}) increased the growth capacity (Figure 12).

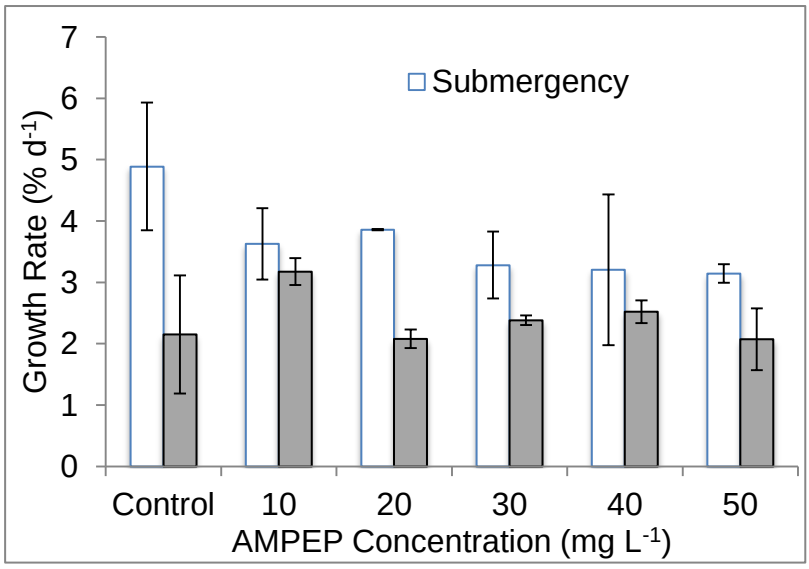

Figure 12. Growth rates of *Gracilaria vermiculophylla* cultivated at six different concentrations of *Ascophyllum* extract, AMPEP (0, 10, 20, 30, 40 and 50 mg /L; n=3) with or without emersion stress. Samples with emersion stress was exposed to air with emersion stress with a 15 min / 1 min emersion/submergence cycle. Error bars represent standard deviation.

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Gorman, L., Kraemer G.P., Yarish C., Boo S.M. and Kim J.K. 2017. The effects of temperature on the growth and nitrogen content of invasive *Gracilaria vermiculophylla* and native *Gracilaria tikvahiae* from Long island Sound, USA. *Algae*, 32(1):57-66.

Kim, J.K. and Yarish C. 2014. Development of a sustainable land-based *Gracilaria* cultivation system. *Algae*. 29: 217-225.

Kim, J.K., Yarish C., and Pereira R. 2016. Tolerances to Hypo-osmotic and Temperature Stresses in native and invasive *Gracilaria* species. *Phycologia*. 55(3): 257-264.

Kim, J.K., Yarish C., Hwang, E.K., Park, M., and Kim, Y. 2017. Seaweed aquaculture cultivation technologies, challenges, and its ecosystem services. *Algae*, 32(1):1-13.

Zhang, J., Kim J.K., Yarish C. and He P. 2016. The expansion of *Ulva prolifera* O.F. Müller macroalgal blooms in the Yellow Sea, PR China, through asexual reproduction. *Marine Pollution Bulletin.* 104:101-106.

Presentation – UCONN

Gorman L., Kim J.K, Yarish C. and Kraemer G. 2015. The effect of temperature on growth of non-native seaweed species *Gracilaria vermiculophylla* in the Long Island Sound as compared to native *Gracilaria tikvahiae.* SUNY Undergraduate Research Conference. The College at Brockport, State University of New York. April 10, 2015.

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2) BARD Project Partner – UCSD, Scripps Institution of Oceanography (SIO):

Objective 2, Establish defined enriched seawater growth media for macroalgae that will induce increased protein content.

Task 2.1: Employ a lab-scale spray culture array at SIO to test the adaptability of the UCONN-supplied strains (*Gracilaria vermiculophylla* and *Ulva lactuca)* to grow under spray culture.

Year-2 - Growth performance: *Ulva lactuca* in the SIO laboratory spray culture system.

Introduction: An economical operation of cultivating Ulva in large-scale land-based systems would be desirable to provide a sustainable source material for protein as an ingredient for aquaculture feed formulation and development, and also as an alternative to ocean cultivation for agar, or biomass for renewable energy. Three different structural cultivation designs for growing *Ulva lactuca* (UCONN strain) in a recirculating seawater spray system in laboratory scale were tested: (1) bag-pocket vertical (BPVD); (2) multi-level horizontal (MLHD); and (3) slant-horizontal design (SHD). Growth rates of seaweed using these cultivation methods were compared with submerged *Ulva* cultures. The interaction effect of cultivation designs used a four-level single factorial randomized block experimental framework ($p<0.05$). In another test, the best performing cultivation design used the four-level two factor experimental design to test interaction effects of cultivation design and Jack's Special Media (JSM) concentrations for growth performance and protein production content of *Ulva*. Results demonstrated that the tested spray system designs showed promising results for improving the growth rate of *Ulva* using MHLD platform and 2X JSM. This indicates that the best performing spray system design can be used for further test in growing *Ulva* in larger**,** pilot production scale (i.e., outdoor greenhouse seaweed cultivation spray systems).

Materials and Methods:

Seaweed culture and stocking density

A mini-spray culture experimental array, in a randomized framework (Figure 1), was employed to perform testing on the UCONN-isolated strains of *U. lactuca* for physiological tolerance to culture protocol factors of light and nutrients using Jack's (commercially – available) Special Media Mix (JACKS). Three replicate polyethylene containers were used per test condition.

Dry Biomass

*Dry biomass = (Wtf * (HWti/Wti)/A)/d (g/m2/day)*

Wtf =Final dry weight

HWti/Wts = wet biomass correction factor (highest wet biomass weight/initial mean weight of sample for each design grouping; initial weight for each grouping with weight difference of 0.01 g)

A= m^2 (0.004 m2, normalized area of sample; 6.35 cm^2)

d = number of cultivation days

Growth Rate (percent growth per day)

% Growth rate = $[(Wt/Wo)^{1/t}-1*100]$

Growth rate (as % GR/day) was calculated using the difference of the final and initial weight divided by the number of days in the culture and expressed as % $[(Wt/Wo)^{1/t}-1*100]$ (Yong et al., 2013). The relative growth rate per day (RGR/day) was computed using the formula (ln Wt-lnWo/t) * 100. (Glen and Doty 1992). Each experimental design-unit contain twelve pieces of ~1 g of pre-weighed seaweed seedlings (total trial = 12). The high number of trials in each replicate unit minimizes the effect of lost branches during seedling preparation and during harvest. Three replicates for each design cultivation unit were used, incorporating three spray system designs and one submerged system design: Multi-level horizontal design (MHLD), (Bag-String Vertical design (BSVD), Netted-Vertical Design (NVD) and S(submerged System) : See photo for graphical representation of each design (Figure 2).

Nutrient Analyses: (see the following website for the nutrient analytical protocol used) https://scripps.ucsd.edu/ships/shipboard-technical-support/odf/documentation/nutrient-analysis

Equipment and Techniques

Nutrient analyses (phosphate, silicate, nitrate plus nitrite, and nitrite) are performed on a Seal Analytical continuous-flow AutoAnalyzer 3 (AA3). After each run, the charts are reviewed for any problems and final concentrations (in micromoles per liter) are calculated using SEAL Analytical AACE 6.07 software.

The analytical methods used are described by Gordon et al. [Gord92], Hager et al. [Hage68] and Atlas et al. [Atla71]. The details of modification of analytical methods used for this cruise are also compatible with the methods described in the nutrient section of the GO-SHIP repeat hydrography manual [Hyde10].

Nitrate/Nitrite Analysis

A modification of the Armstrong et al. [Arms67] procedure is used for the analysis of nitrate and nitrite. For nitrate analysis, a seawater sample is passed through a cadmium column

where the nitrate is reduced to nitrite. This nitrite is then diazotized with sulfanilamide and coupled with N-(1-naphthyl)-ethylenediamine to form a red dye. The sample is then passed through a 10mm flow-cell and absorbance measured at 540nm. The procedure is the same for the nitrite analysis but without the cadmium column.

Phosphate Analysis

Ortho-Phosphate is analysed using a modification of the Bernhardt and Wilhelms [Bern67] method. Acidified ammonium molybdate is added to a seawater sample to produce phosphomolybdic acid, which is then reduced to phosphomolybdous acid (a blue compound) following the addition of dihydrazine sulfate. The sample is passed through a 10mm flow-cell and absorbance measured at 820nm.

Protein Content of Spray cultured Biomass

Dry samples (10-20 mg each) from each of the growout trials were packaged and sent to the UC Santa Barbara analytical facility for elemental analysis (carbon, hydrogen, nitrogen). From the nitrogen content reported, protein will be calculated using a nitrogen to protein multiplyer of 5.65 as reported in the literature for cultured *Ulva lactuca* biomass (see: Shuuluka et al. 2013).

Sampling Requirements and Preservation

Freezing is the preferred method of preservation. 20-30 mL of sample are required for analysis. Polypropylene screw-capped centrifuge tubes are recommended. Tubes should be sterile or washed thoroughly with 10% HCl and rinsed with sample at least 3 times prior to filling. Leave a headspace for expansion during freezing. Samples are thawed overnight at ~1.7 C and brought to room temperature prior to analysis. The centrifuge tubes fit directly onto the sampler.

Data Collection and Processing

Data collection and processing is done with the software (ACCE ver 6.07) provided with the instrument from Seal Analytical. After each run, the charts are reviewed for any problems during the run, any blank is subtracted, and final concentrations are calculated, based on a linear curve fit. Next, a text file is created. This is reviewed for possible problems and then

converted to an output file with only sample identifiers and nutrient concentrations that can be merged with other bottle data.

Standards and Glassware Calibration

Primary standards for silicate (Na2 SiF6), nitrate (KNO3), nitrite (NaNO2), and phosphate (KH2 PO4) are obtained from Johnson Matthey Chemical Co. and/or Fisher Scientific. The supplier reports purities of >98%, 99.999%, 97%, and 99.999 respectively.

All glass volumetric flasks and pipettes are gravimetrically calibrated. The primary standards are dried and weighed out to 0.1 mg. When primary standards are made, the flask volume at 20°C, the weight of the powder, and the temperature of the solution are used to buoyancy correct the weight, calculate the exact concentration of the solution, and determine how much of the primary is needed for the desired concentrations of secondary standard. New standards are compared to the old before use.

All the reagent solutions, primary and secondary standards are made with fresh distilled deionized water (DIW).

Standardizations are performed at the beginning of each group of samples.

Kinetics of Nutrient Uptake

The steady state kinetics of nutrient uptake and seaweed growth is successfully used in the course of alga cultivation (Silkin et al., 1992 ; Silkin et al., 2007) on the basis of the regulation of uptake process is the dependence of the uptake rate of the nutrient concentration in the medium that can be expressed by the equation of Michaelis-Menten:

$\upsilon = \upsilon_{max} \times C/K+C$

where C is the nutrient concentration in the medium; υ and υ_{max} is the specific and maximum specific uptake rate; and K is the half saturation constant , equal to the nutrient concentration, when $\upsilon=\upsilon max/2$, µmol.

Spectrophotometric Pigment measurement and calculation

Pigments were extracted from seaweeds using mortar and pestle in 90% acetone. Spectrum of extracted pigment were determined by a Varian Spectrophotometer. Chl a, chl b and carotene were calculated as follows:

Chl a (mg g-1) = [(11.75× A662) - (7.340× A645)] × V(ml) / mg seaweed tissue x1000
Chl b (mg g-1) = [(18.61 × A645) - (3.960 × A662)] × V(ml) / mg seaweed tissue x1000
Total Chl = Chl a + Chl b
*Carotenoid (mg/g) = 1000*A480 -(2.270 × chla) – (81.4 × chlb)/227*

Statistical Analyses of Results Obtained

Normality of data were tested using Shapiro-Wilkinson test and Brown-Forsythe for variance equality test. The effects of the culture conditions and JSM concentrations on the growth rate were verified through univariate general linear model. This was followed by Holm-Sidak method (significance level of 0.05) for the pairwise multiple comparison.

1. Results

The MHLD showed higher mean daily biomass gain (~16g/m2/day) among the three spray system platform with the 2X JSM concentration (Figure 3). The growth rate of *U.lactuca* in MHLD platform with 2X JSM, exhibit a maximum of ~9%/day. With 80% water content, the estimated ash-free dry weight of *Ulva* on MHLD platform reached 13.18 g/m2/day, which is half the *Ulva* AFDW on the SUB platform.

From the nutrient data, uptake rates of Ulva were estimated using the Michaeles-Menten equation at different JSM concentrations (Table 2). Ulva on the spray system platforms (SHD, MHLD, BPVD) exhibits relatively higher uptake than the SUB. This is true for nitrate, ammonia, dissolved inorganic nitrogen and phosphate (Figure 5, 6, 7, 8). Ulva on platforms MHLD and SHD have higher preference for nitrate uptake, while BPVD have more preference for ammonia and phosphate. As shown in the kinetic parameters, Ulva's maximum uptake rate and half-saturation value of nitrate on MHLD and SHD platforms is closely similar (Table 3). The use of different *Ulva* cultivation platforms effect uptake rates of these nutrients.

The difference in the mean dry biomass/day among the different levels of JSM is greater than would be expected by chance after allowing for effects of differences in the platform of cultivation and the different JSM levels: there is an observed statistically significant difference (P = <0.000) (Table 4). To isolate which group(s) differ from the others, a univariate multiple pairwise comparison procedure employed showed highest difference of dry biomass/day at 2X JSM with significant variation with 1X followed by 8X JSM (Table 5). Note that these mean difference comparison is significant at 0.05 levels. The JSM concentration and its interaction with the design as the factor showed significant effect on the mean dry biomass production per day of *Ulva lactuca* suggesting that specific nutrient levels is a controlling factor that led to the increase or decrease of the seaweed's growth rate.

Pigments analysis (Chl a, Chl b and carotene) were highest in the SHD platform, even better than SUB. This means that the pigments are not necessarily the controlling factor the directly affect the biomass build-up in *U. lactuca*.

The monitored pH, temperate, and light exhibits slight variations (Table 7). These co-variates will be considered in the correlation analysis of all treatments and variables with the protein production once we have the finalized protein production data.

Results for protein content of spray cultured biomass have not - as of this writing, been received from the UCSB analytical laboratory (due to administrative delays beyond our control). Those results will be reported in the subsequent project report, and to the partners when finally received from UCSB.

Summary of Results

The Multi-level Horizontal design (MLHD) provided the highest growth rate of *Ulva lactuca* in a recirculating system using 2X Jacks Special Media (JSM). However, we concluded that the best growth rate can be obtained using glass type of material rather than using PE plastic containers. Based on these experimental results we project that improved growth rates and protein content should be obtainable under outdoor, higher solar light conditions as would be found in a greenhouse environment. We will further explore other input parameters (e.g., CO_2) to maximize growth and protein production of *Ulva lactuca* in spray recirculating system.

Development of new markets for cultured macroalgae will promote new environmentally friendly seaweed culture businesses (i.e., development of seaweed based shrimp aqua-feeds will increase the sustainability of the intensive shrimp culture industry and in for advancing inland aquaculture of shrimp.

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FIGURES (SIO – Yr 2):

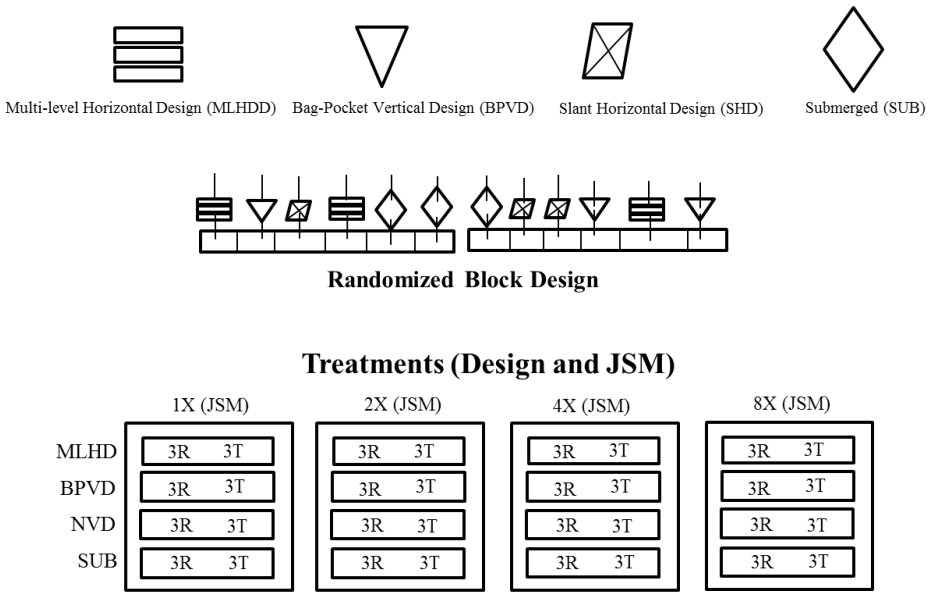


Figure 1. An randomized experimental design was employed to test the design and JSM concentration with growth performance of *Ulva* at alpha = 0.05. The study uses a four level two-factorial experiment to determine main effects and the interaction of these factors.

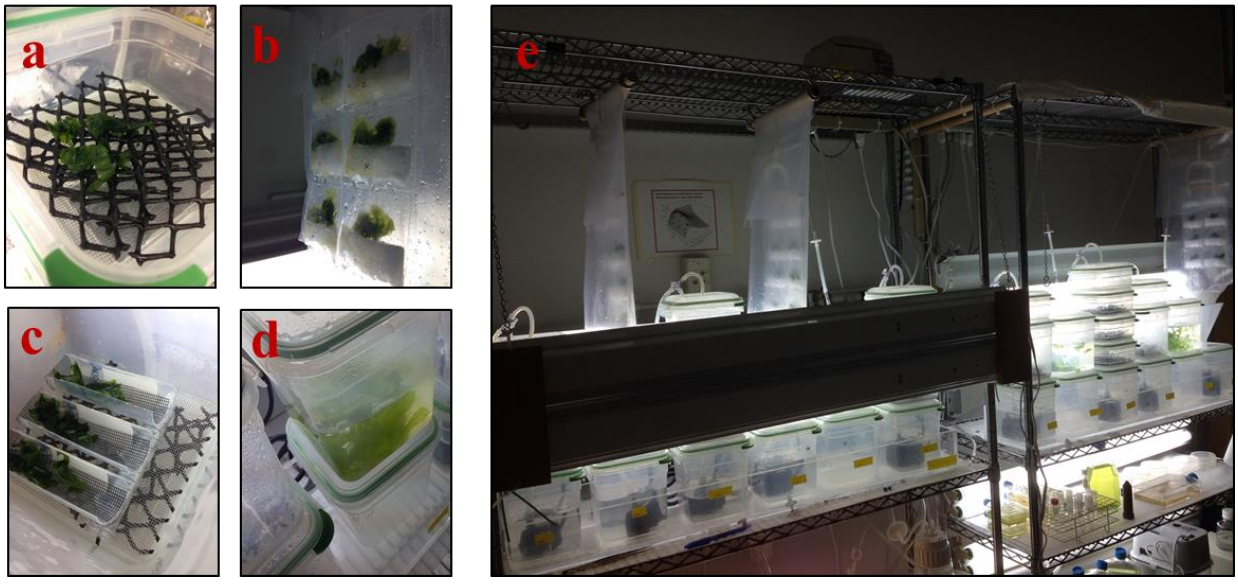


Figure 2. . Seaweed cultivation platform of *Ulva lactuca* grown at different JSM (mean ± stdev, n=3) concentrations on three different cultivation spray system for about 15 days. a) Multi-Level Horizontal Design (MHLD); b) Bag-Pocket Vertical Design (BPVD), c) Slant-Horizontal Design (SHD), d) Submerged (SUB).

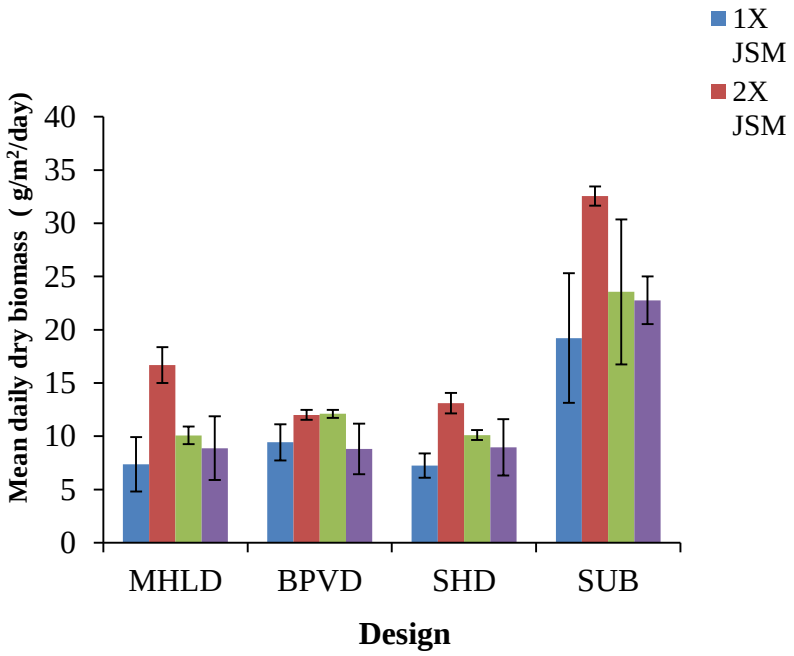


Figure 3. Mean biomass per day of *Ulva lactuca* grown at different JSM (mean ± stdev, n=3) concentrations on three different cultivation spray system cultivation for about 15 days.

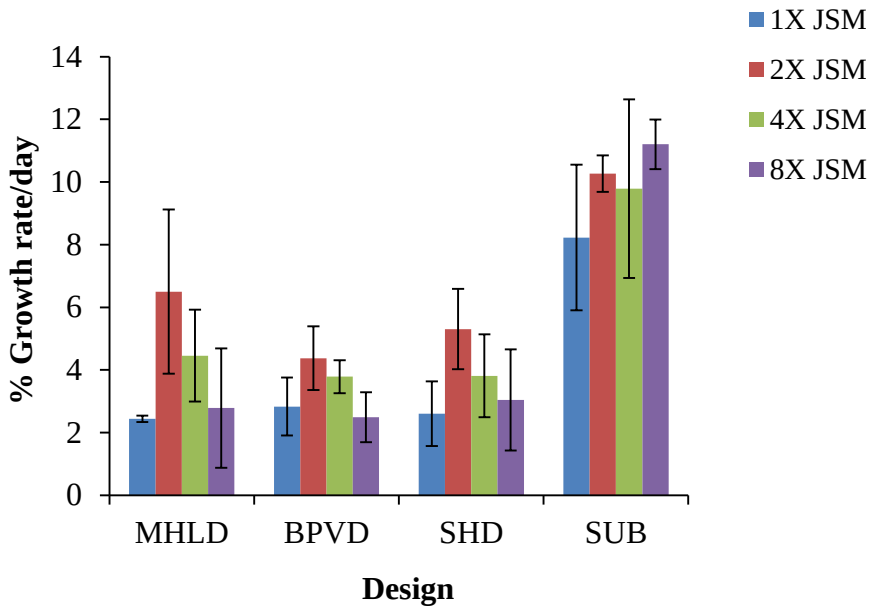


Figure 4 . %Growth rate per day of *Ulva lactuca* grown at different JSM (mean ± stdev, n=3) concentrations on three different cultivation spray system cultivation for about 15 days.

Table 1. Water and AFDW content (ash-free dry weight) of *U. lactuca* (mean ± stdev, n=3) grown on different cultivation design-units (MLHD, BPVD, SHD and SUB) at different JSM concentrations.

Design	**Water content**	**AFDW**
	(%)	*g/m^2/day*
1X		
MHLD	74.53±4.95	5.72±0.14
PBVD	76.26±2.83	7.17±0.08
SHD	77.87±1.35	5.92±0.05
SUB	77.75±0.87	14.05±0.36
2X		
MHLD	77.37±1.19	13.18±0.02
PBVD	76.14±0.86	9.24±0.01
SHD	78.92±0.94	10.59±0.04
SUB	73.39±0.96	26.43±0.02
4X		
MHLD	79.67±1.37	7.93±0.02
PBVD	75.60±0.64	9.34±0.02
SHD	80.54±0.84	7.89±0.00
SUB	75.77±1.06	17.86±0.09
8X		
MHLD	81.17±2.65	6.98±.10
PBVD	75.34±5.32	7.22±0.10
SHD	78.62±3.18	7.85±0.08
SUB	80.06±0.83	17.16±0.04

Table 2. Nutrient concentrations of JSM on three different cultivation design-units (MLHD, BPVD, SHD and SUB).

Design	**NO3**	**NH4**	**DIN**	**PO4**
	µM	*µM*	*µM*	*µM*
Initial				

1X	252	166.8	419.2	30.8
2X	502	329.2	831.6	60.4
4X	1008	667.2	1676.8	123.2
8X	2440	1652.8	4099.2	328
SHD				
1X	0.27±0.04	0.26±0.01	0.55±0.06	2.10±0.57
2X	0.37±0.07	0.52±0.16	0.90±0.22	2.68±0.23
4X	87.77±15.49	2.31±0.80	90.76±14.25	11.84±1.17
8X	271.80±88.95	58.09±13.85	330.38±76.59	32.49±8.62
MHLD				
1X	1.03±1.121	1.42±1.17	5.40±6.11	15.39±12.20
2X	21.29±29.72	0.57±0.51	21.95±30.36	4.86±4.34
4X	67.27±5.48	1.44±0.58	47.01±37.91	5.49±4.84
8X	302.52±98.42	88.87±78.59	392.67±177.99	37.34±16.07
PBVD				
1X	1.44±0.51	1.54±0.42	2.98±0.94	19.44±7.27
2X	57.79±61.813	3.38±0.41	61.49±61.75	29.80±2.60
4X	615.10±206.30	7.35±0.62	625.66±206.94	66.84±22.39
8X	1861.59±347.60	214.28±57.39	2081.68±292.61	50.67±1.35
SUB				
1X	0.57±0.43	0.47±0.33	1.06±0.77	1.10±1.58
2X	1.69±0.56	2.06±0.64	3.87±1.23	5.70±1.89
4X	2.48±0.35	4.32±1.09	55.73±85.84	12.94±17.49
8X	7.42±1.61	497.73±40.34	508.19±39.12	2.78±0.20

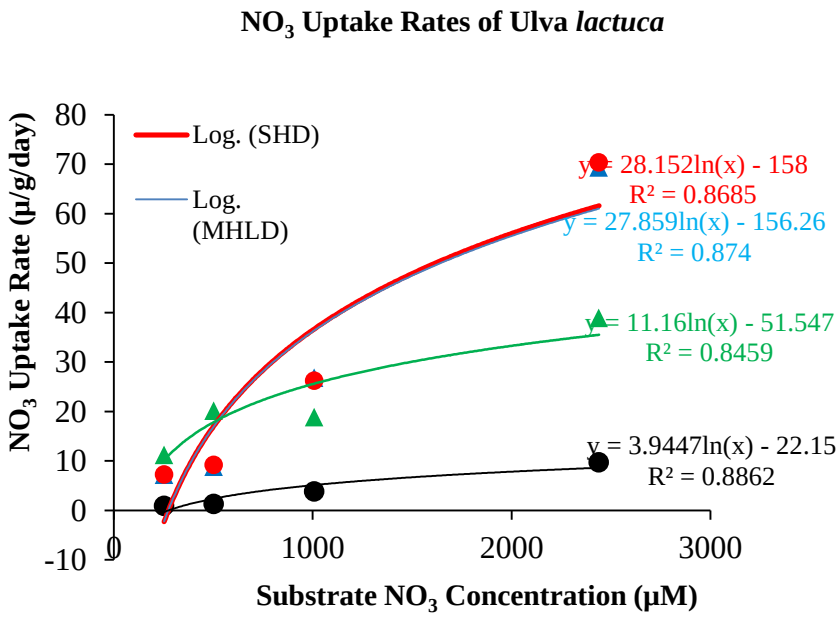


Figure 5. Parameters of the Michaelis–Menten equation estimated by non-linear fitting of data from the four NO_3 uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

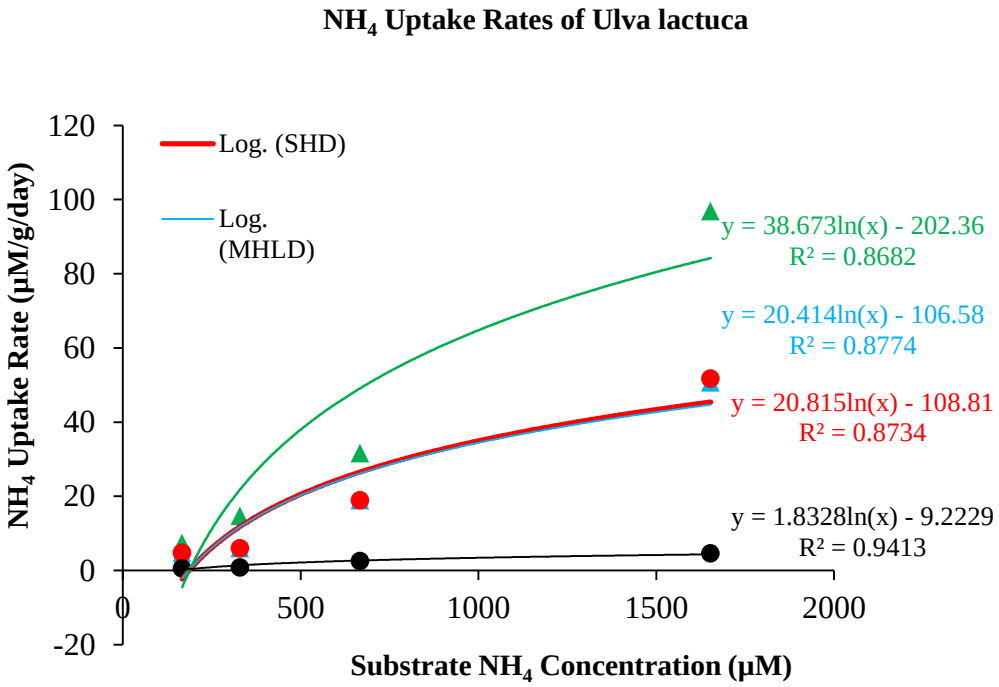


Figure 6. Parameters of the Michaelis–Menten equation estimated by non-linear fitting of data from the four NH_4 uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

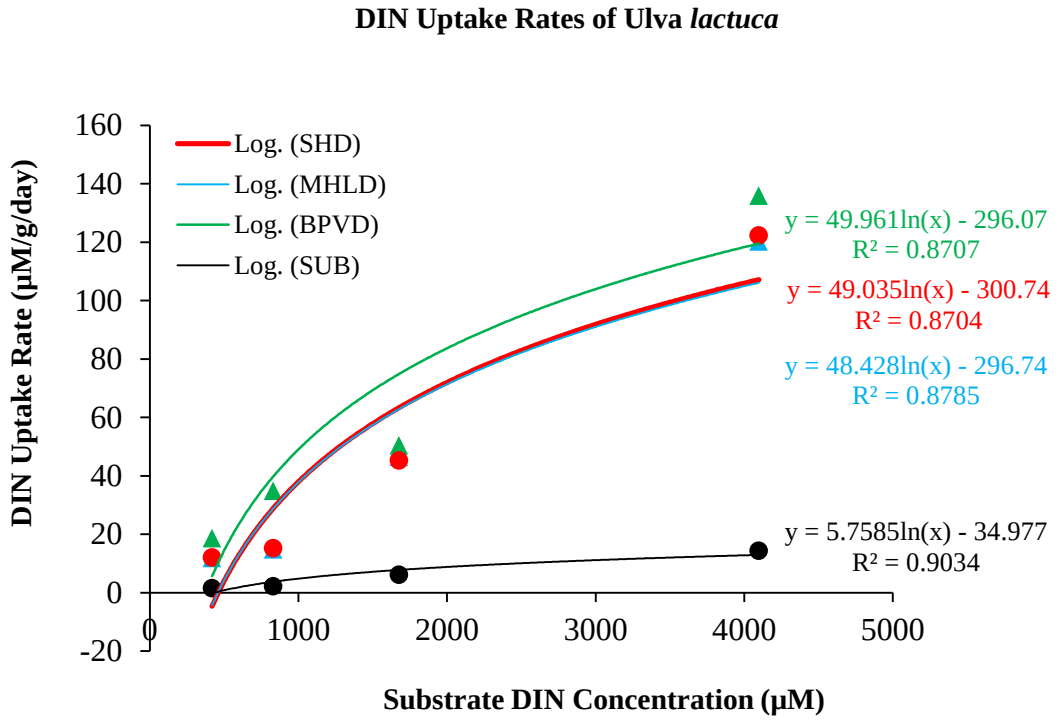


Figure 7. Parameters of the Michaelis–Menten equation estimated by non-linear fitting of data from the four DIN uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

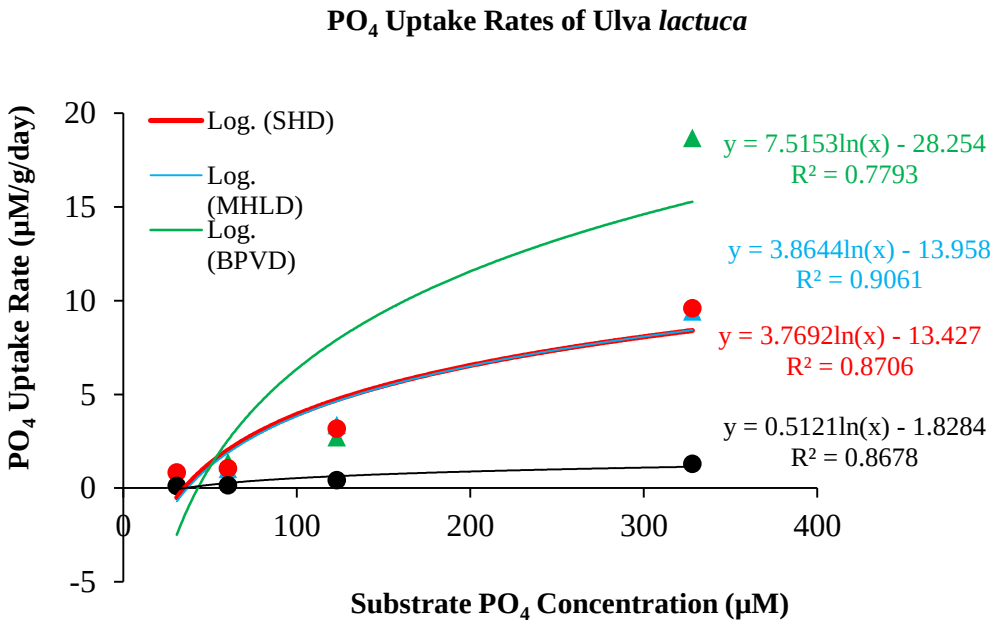


Figure 8. Parameters of the Michaelis–Menten equation estimated by non-linear fitting of data from the four PO_4 uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

Table 3. Kinetic parameters V_{max} (µmol N (g DW)$^{-1}$ h^{-1}), K_m (µM N) and affinity for uptake at low concentrations (V_{max}/K_m) for nitrate, ammonia, dissolved inorganic nitrogen and phosphate uptake in *U. lactuca*.

Design	NO_3			NH_4			DIN			PO_4		
	Vmax	*Km*	$\frac{VMax}{Km}$	*Vmax*	*Km*	$\frac{VMax}{Km}$	*Vmax*	*Km*	$\frac{VMax}{Km}$	*Vmax*	*Km*	$\frac{Vmax}{Km}$
SHD	61.57	817.35	0.075	45.43	554.94	0.082	107.16	1374.52	0.078	17.93	380.09	0.047
BPVD	35.51	497.27	0.071	84.22	553.12	0.081	119.53	1239.28	0.096	34.26	419.49	0.082
MHLD	61.03	815.95	0.075	44.69	556.36	0.151	106.11	1370.59	0.077	18.19	389.65	0.047
SUB	7.16	776.19	0.009	4.36	503.30	0.009	12.93	1334.43	0.010	2.43	381.64	0.006

Table 4. Univariate general linear models comparing interaction of effects of cultivation design and JSM concentration. The F tests the effect of 1. This test is based on the linearly independent pairwise comparisons among the estimated marginal means. Computed using alpha = 0.05.

	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Contrast	392526.83	3	130842.28	24473.14	.000	.610
Error	251444.76	47031	5.35			

Table 5. Pairwise comparison using univariate general linear models comparing interaction of effects of design and JSM concentration.

Dependent Variable: Dry Biomass ($g/m^2/day$)

(I) 1	(J) 1	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
1	2	-7.964*	.031	.000	-8.025	-7.904
	4	-3.783*	.031	.000	-3.843	-3.722
	8	-2.158*	.031	.000	-2.218	-2.098
2	1	7.964*	.031	.000	7.904	8.025
	4	4.182*	.030	.000	4.123	4.240
	8	5.806*	.030	.000	5.748	5.865
4	1	3.783*	.031	.000	3.722	3.843
	2	-4.182*	.030	.000	-4.240	-4.123
	8	1.625*	.030	.000	1.566	1.683
8	1	2.158*	.031	.000	2.098	2.218
	2	-5.806*	.030	.000	-5.865	-5.748
	4	-1.625*	.030	.000	-1.683	-1.566

Based on estimated marginal means.* The mean difference is significant at the .05 level.

[b]Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 6. Chl a, chl b, total Chl and total carotene (mean ± stdev, n=3) grown in different cultivation design-units (MLHD, BPVD, SHD and SUB).

Design	**Chl a**	**Chl b**	**Total Chl**	**Total Carotene**
	mg/g	*mg/g*	*mg/g*	*ug/g*
1X				
MHLD	0.40±0.29	0.39±0.39	0.79±0.58	0.76±0.38
PBVD	0.37±0.34	0.35±0.33	0.72±0.67	0.76±0.30
SHD	0.67±0.38	0.65±0.31	1.31±0.69	0.78±0.25
SUB	0.13±0.12	0.14±0.14	0.27±0.26	0.40±0.23
2X				
MHLD	0.24±0.19	0.23±0.18	0.48±0.37	2.59±1.52
PBVD	0.13±0.05	0.12±0.04	0.25±0.09	1.56±0.41
SHD	0.29±0.11	0.27±0.10	0.56±0.21	1.96±0.39
SUB	0.14±0.07	0.13±0.06	0.27±0.13	3.14±1.81
4X				
MHLD	0.77±0.24	0.76±0.27	1.53±0.51	1.64±0.48
PBVD	0.93±0.02	0.86±0.03	1.79±0.06	1.39±0.39
SHD	1.01±0.38	0.90±0.36	1.91±0.74	1.20±0.12
SUB	0.49±0.15	0.49±0.15	0.98±0.30	0.52±0.06
8X				
MHLD	0.07±0.09	0.06±0.08	0.13±0.17	0.34±0.51
PBVD	0.64±0.19	0.60±0.17	1.24±0.36	0.66±0.49
SHD	0.90±0.43	0.83±0.40	1.73±0.83	0.80±0.56
SUB	0.74±0.08	0.66±0.09	1.40±0.17	1.58±0.66

Table 7. Culture conditions of *Ulva lactuca* grown in three different cultivation designs.

		pH	**Temp (^{o}C)**	**Light ($\mu E/m^2/s$)**
1X	*MHLD*	8.42±0.01	26.57±0.21	202.00±48.48
	BPVD	8.47±0.03	25.90±0.40	157.73±16.83

	SHD	8.36±0.09	27.10±0.61	218.61±60.05
	SUB	9.05±0.72	26.23±0.68	251.81±31.15
2X	*MHLD*	-	27.27±0.64	118.07±3.20
	BPVD	-	26.30±0.44	136.51±27.86
	SHD	-	27.27±0.51	146.66±33.55
	SUB	-	26.77±0.40	140.20±36.85
4X	*MHLD*	8.59±0.09	28.80±0.26	196.57±17.29
	BPVD	8.57±0.13	26.63±0.15	164.27±26.89
	SHD	8.46±0.03	28.67±0.32	157.81±54.46
	SUB	9.98±0.13	25.90±0.53	219.64±6.39
8X	*MHLD*	8.01±0.17	29.50±0.53	175.25±20.95
	BPVD	7.81±0.12	27.47±0.57	171.56±5.53
	SHD	7.89±0.16	28.57±0.75	199.24±28.76
	SUB	9.69±0.17	25.93±0.40	215.84±59.86

3) Partner Organization: Israel Oceanographic and Limnological Research, National Center for Mariculture (IOLR-NCM)

Objective 3 (re-stated). Cultivation of several seaweed species at kg quantities (dw) for manufacture of seaweed meal for use in experimental shrimp diets to be performed by Auburn, USA partner.

In Israel, the two best performing strains from UCONN/UCSD-SIO testing (plus a strain selected locally) will be grown in larger spray-culture units in a greenhouse (to be constructed at NCM), and tested against each other for overall growth and biomass performance, and protein-lipid spectrum and content. Seawater and nutrients for the spray-culture growth trials will be supplied from near-by fish ponds and piped/pumped to the spray-culture greenhouses. Use of strains from the USA is justified, since it can be expected that deployment of the developed technology will begin in the USA, and will be contained in land-based cultivation units. Controlled algal biomass production of the greenhouse-cultured macroalgae will be conducted using seawater, enriched by either fertilizers or fish-pond/shrimp-pond effluent.

This year's efforts at NCM, Eilat have made further progress toward the objectives:

8. The experimental outdoor spray irrigation system, which was completed and reported on in our 2014 year-end report, produced silty and protein deficient algae. This was due to the unavailability of sediment-free nutrient-rich fishpond effluents. Following a consultation with Prof. Samocha (TAMU), a water filtration nutrient- enrichment system was designed and built to supply nutrient-rich non-silty algae biomass for our 2015 experiments (Figure 1).

9. The modified system began operation in the spring of 2015, and the algae proved to be of better quality than in the 2014 batches. Production was lower then expected, due to a relatively difficult winter season, and some unforeseen hardware failures. Overcoming the failures and cooler winter temperatures by the spring allowed for better growth in the summer months, and by the end of summer, NCM made a shipment of approximately 7 kg of *Ulva* sp. (green seaweed) Meal to Auburn Univ. This biomass assayed with a higher protein content then contained in the 2014 batches, and is currently being characterized by Auburn for incorporation into a shrimp feed experimental diet for feeding trails upcoming. A second shipment of a similar size is planned for shipment in October, 2015. Total Ulva meal prepared to-date will reach approximately 15 kg d.w. (Table 1).

10. A novel tile-based drip irrigation unit has been designed and built in a greenhouse, following its renovation in summer 2014 (Figure 2). It is a somewhat smaller system (overall area and capacity) than the first outdoor system. However, its location in the greenhouse and its connection to the supply of nutrient-enriched fishpond effluents should facilitate production of a winter crop of Ulva.

11. The algal species brought to NCM by Prof. Yarish from UCONN (an *Ulva* (green seaweed) and two *Gracilaria* species (red seaweed); see UCONN report herein included) - were placed for growth in an NCM not temperature controlled culture room. While at first both algae grew, in early summer both cultures died, apparently due to the intense summer heat in the non air-conditioned growth room.

12. Our associate, Yossi Bronfman - who has been studying for an MSc degree in the Hebrew University of Jerusalem, has completed experiments on cultivation of several macroalgae species in spray culture. Below is a brief report on these experiments:

a. Background:

Two different prototypes of spray culture systems were built. The water supply (i.e. spray) in both prototypes was consisted of on long narrow tubes, with tiny pores. The tubes were placed at the top of the culture trays. The water dripped over the upper part of the tray and flowed down, covering and wetting the algae. This was better for *Ulva* spp. then for red algae, where water sprinkling over the entire tray was necessary.

b. Main research questions:

i. Evaluations of locally-available species is for spray culture cultivation, and their performance.

ii. Optimization of the nutrients uptake rates from fish ponds effluent.

c. Approaches

i. Optimal slope and the resulting stream velocities over the diffusion boundary layer of the algae.

ii. Optimization of the nutrient load.

d. Methods

i. The system in Mevo'ot Yam School in Michmoret, described in the 1st year report: The culture units of plastic trays, size of 0.12 m^2, water-sprayed at 100L/hr per tray. The water then drained into drainage tank and then pumped back up into the influent header. Water residence time in the system was 12 hr.

***Ulva fasciata* grew in three culture systems:**

A. A regular aerated pond (Control).

B. A horizontal (0^{0-}inclined) tray that provided a thin layer of water. Water spilled equally from all sides of the tray.

C. A tray with a slope (6^0 slope from the horizontal), algae were submerged in water but not completely covered aeration. Water dripped on top and flowed downhill, to spill away from the lower edge.

After each test, the algae were rinsed, weighed and dried (60 ^{0}C for 48h).

Samples were prepared for Ash, Kjeldahl protein, phosphorus and lipids. Records were kept of temperature, pH, sunlight and photosynthetic quantum yield (PAM fluorometer). Water samples were collected for nutrients content. Experiments were made first with fresh sea water, then with the enrichment to a load of by 10 gr ammonia-N and 1 gr phosphate-P 1 m^{-2} d^{-1}.,

The algae were monitored and records taken for appearance, epiphyte cover and additional parameters. Calculation of yield = $(W_t - W_0)$ /t/ m^2. t = days of experiment, W_0 = initial weight, W_t = Weight at the end after (t) days

$$\text{Calculation of SGR} = \frac{\ln\left(\frac{wt}{w0}\right) x\ 100}{t}$$

Calculation of ash-free protein content= (Protein percentage*100)/(100-Ash percentage)

ii. A spray culture system at IOLR, Haifa (Figure 3)

This series of experiments was dedicated to fine-tuning the spray culture method, vsv slope and nutrient fertilization.

The effect of different tray slope/stream-velocities on the algae nutrients accumulation ability was evaluated in more detail.

Tray angles examined were 0^0, 6^0, 40^0 and 80^0. We also considered the impact of slope on light intensity.

Several levels of nutrient enrichment were evaluated with *Ulva fasciata* on 6^0 trays: no additional fertilizer, and with the addition of 5, 10, 15 g N and 0.5, 1, 1.5 g P m^{-2} d^{-1}, respectively. A control was enrichment of 1 g N and 0.1 g P m^{-2} d^{-1} in a tank of same surface area as a tray.

This setup was applied to *Ulva fasciata*, (green seaweed); *Hypnea mosciformis* (red seaweed) and *Ulva compressa* (green seaweed).

Experimental:

Each 0.12 m^2 t ray was sprayed on top at 500 L m^{-2} h^{-1}. Source of seawater as a pump placed in the sea at 5m depth. Nutrients were added by gravity directly to the water supply pipe.

e. Results (selected main findings); most of the chemical data are still in production.

i. Measured stream velocity over the algae-loaded trays was 6 times higher at 80^0 than at 0^0 .

ii. Slope impacted both yield and Kjeldahl protein content in ash free DW. Protein content and growth rate (Table 1) were lower in the spray-cultured algae than in the initial and control (tank-grown) biomass.

iii. Fertilization impacted positively both yield and protein content in Ulva (Table 2).

iv. Fertilization had a mixed impact on yield (data on protein content are not yet available) in *Hypnea mosciformis* (Table 3). But the absolute value of the yield was high.

f. Tables (NCM)

Table 1: *Ulva* sp. meal preparation in 2015.

Harvest date	**Milled weight, kg**
21.5.15	1.58
28.5.15	1.904
3.6.15	1.418
18.6.15	0.418
20.7.15	1.574
30.7.15	0.724
2.8.15	0.408
4.8.15	0.278
16.8.15	0.938
20.8.15	0.684
23.8.15	1.258
25.8.15	1.904
30.8.15	0.998
2.9.15	0.681
1.7.15	0.178
TOTAL	14.945

Table 2: Yield and Kjeldahl protein in ash free DW in *Ulva fasciata* as a function of tray slope. Control in a normal pond.

Tray slope	Yield g FW m^{-2} d^{-1}	Kjeldahl protein % in AFDW
Initial biomass		29.5
0°	66.6	20.3
6°	75.5	21.4
40°	58.3	22.4
80°	41.7	24.5
Control	124.2	22.0

Table 3: Yield and Kjeldahl protein in ash free DW in *Ulva fasciata* as a function of nutrient fertilization. Fer 1, 2, 3 = 5, 10, 15 g N and 0.5, 1, 1.5 g P m^{-2} d^{-1}, respectively, twice a week. Control = 10 g N and 1 g P m^{-2} d^{-1}. Control in a normal pond.

Treatment	Yield g FW m^{-2} d^{-1}	Kjeldahl protein % in AFDW
Initial biomass		15.44
No Fer	20	11.75
Fer 1	64	24.06
Fer 2	77	23.60
Fer 3	84	26.47
Control	100	26.46

Table 4: Yield in *Hypnea mosciformis* as a function of nutrient fertilization. Fertilization treatments were as in table 2.

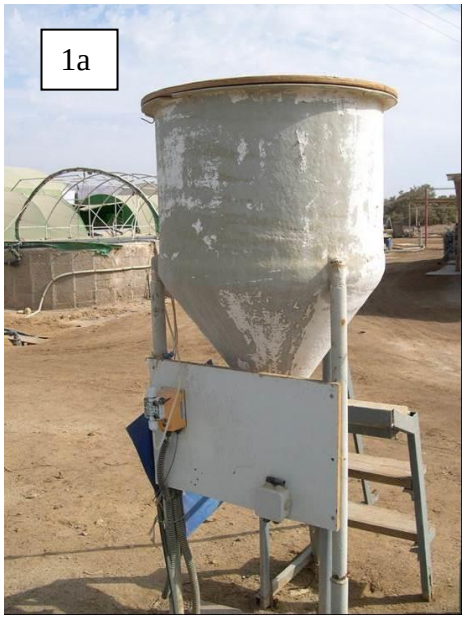
Treatment	Yield g FW m^{-2} d^{-1}
Initial biomass	
No Fer	200
Fer 1	197
Fer 2	286
Fer 3	248
Control	318

Table 5: Yield and Kjeldahl protein in ash free DW in *Ulva compressa* as a function of nutrient fertilization. Fertilization treatments were as in table 2.

Treatment	Yield g FW m^{-2} d^{-1}
Initial	
No Fer	61
Fer 1	75
Fer 2	110
Fer 3	194
control	95

6. Figures (NCM)

Figure 1: A water filtration / nutrient-enrichment system.
1a, a 400 L nutrient solution tank with a dosing pump.
1b, an agricultural water filter (100 micron mesh).



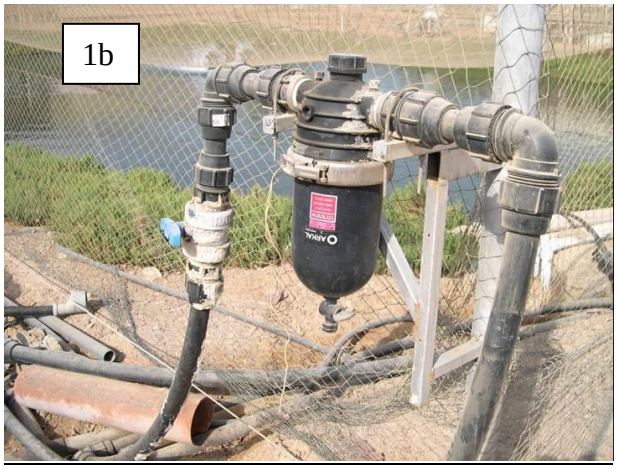


Figure 2: A tile drip-irrigation culture system in a greenhouse in Eilat, Israel. Three oval fiberglass ponds, each containing eight cement tiles, each tile with a culture surface area of 0.2 m^2. (2a), the entire system; (2b), at stocking, May 2015; (2c), after 2-weeks of growth.

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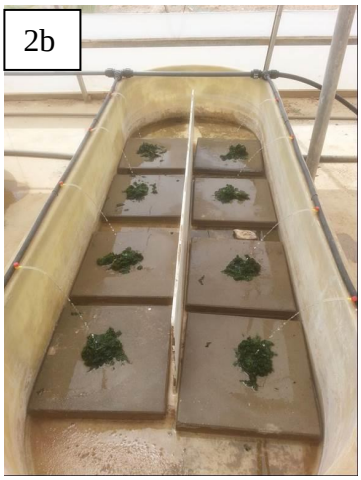

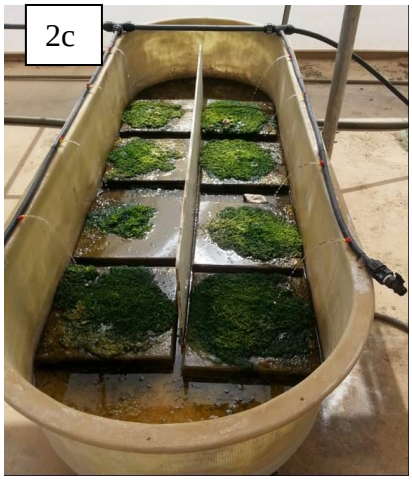

Figure 3: Spray culture system in Haifa, with *Ulva fasciata*. In left upper side of the picture the small nutrients pipe and the regulator can be noticed.

4) BARD Project Partner – Auburn University, Department of Fisheries and Allied Aquacultures (AUBURN)

Overview

Due to the unforeseen institutional shut-down of shrimp culture facilities at Texas A & M's Flour Bluff Experiment Station, years two and three of the Texas A&M Agrilife component of the BARD project were transferred to Auburn University, School of Fisheries, Aquaculture and Aquatic Sciences. Contracting and account development were completed and the appropriate paper work completed to move the project forward. The Auburn component has

been headed by Fisheries and Aquaculture Nutrition Professor, Dr. D. Allen Davis, who assigned an Auburn School of Fisheries PhD student to the project.

Dr Amir Neori, Israel Oceanographic and Limnological Research Ltd, National Center for Mariculture, Eilat, Israel provided approximately 10 kg of dried *Ulva* spp. powder for testing. This meal was used for growth and digestibility trials with juvenile (5-10 gram) Pacific white shrimp *L.vannamei*.

Objective 4. Design and preparation of experimental shrimp diets based on the composition of commercial aquaculture diets and the composition of the dried seaweed product from Israel.

Dr. Tzachi Samocha (TAMU) transferred information obtained in year one of the project and we have reviewed that information. A sample of *Ulva* powder was provided for which the nutrient content is being confirmed. AUBURN sourced all the required ingredients and verified their nutrient content for the shrimp diets for the feeding trials. Based on typical commercial production diets for this species, a basal diet for digestibility (Table 1) and a practical basal diet for ingredient testing was developed (Table 2). With the exception of the reference diet for digestibility, all test diets were formulated to have equal protein and lipid levels (35% protein and 8% lipid), and designed to meet known nutrient requirements for this species. Diets were prepared in the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University (Auburn, AL, USA) using standard procedures. In short, primary ingredients were analyzed for composition and then the diets will be formulated. Pre-ground dry ingredient and oil will be mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then be blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air dried (<50 °C) to a moisture content of 8-10%. After drying, pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed.

Table 1. Composition of reference diet for the determination of digestibility coefficients of algae meal. The basal diet and test ingredient will me mixed using 70:30 ration (dry matter basis) to produce the test diets.

Ingredient	g/100g
Menhaden fish meal	10.00
Soybean meal	32.50
Menhaden Fish Oil	3.20
Corn Starch	2.10
Whole wheat	47.60
Trace Mineral premix	0.50
Vitamin premix w/o choline	1.80
Choline chloride	0.20
Stay C	0.10
Lecithin	1.00
Chromic oxide	1.00

Table 2. Composition (g/100g as is) of the proposed test diets for the growth trial. Final formulations are subject to change based on the actual composition of the meals.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Menhaden fish meal	10.00	8.00	6.00	4.00	2.00	0.00	0.00
Soybean meal	48.00	48.00	48.00	48.00	48.00	48.00	48.00
Corn protein concentrate	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Menhaden fish oil	5.49	5.62	5.75	5.89	6.02	6.15	6.15
Corn starch	13.21	10.54	7.90	5.25	2.63	0.00	0.08
Whole wheat	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Trace Mineral premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix w/o choline	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C 250 mg/kg	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Calcium phosphate	1.62	1.90	2.15	2.40	2.65	2.90	2.90
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Methionine	0.03	0.04	0.05	0.06	0.07	0.08	0.00

Ulva Powder 30% Protein	4.25	8.50	12.75	16.98	21.22	21.22

Objective 5. Shrimp feeding trials with the experimental seaweed diets in comparison with a commercial aquaculture shrimp diet.

AUBURN obtained post larval *L. vannamei* shrimp, and reared them at the Claude Peteet Mariculture Center in Gulf Shores, AL. The PL shrimp are expected to be of suitable size (ca. 5-10 grams) to initiate a growth trial at the end of September, 2015. At that time, the diets for the growth trial will have been finalized and produced. The test diets, for the determination of digestibility will be made for inclusion in a digestibility trial that will be run after the conclusion of the growth trial. Subsequently, everything was put into place to initiate the growth and digestibility trial during the last quarter of this year (2015).

Project Cooperation, Changes in Project Direction & Allied Activities

(UCONN): Drs. Yarish and Kim visited UCSD / Scripps Institution of Oceanography (**SIO**) as part a bilateral program between NOAA and The *National Fisheries Research and Development Institute* (*NFRDI*) of Korea on Mar. 23rd, 2015. Drs. Yarish and Kim discussed seaweed cultivation systems and experimental design for the BARD project with project PIs, Drs. B. Greg Mitchell and Dominick Mendola, as well as the post-doctoral associate, Dr. Wilson Mendoza of UCSD-SIO.

(UCSD-SIO): Considerable cooperation was required and obtained between the **TAMUS** partner, the new Auburn Univ. partner (**AUBURN**) and **SIO**, the overall project P.I., project manager and coordinator, in order to transfer the **TAMUS** responsibilities and remaining funding resources over to Auburn, due to the pending closure of the **TAMUS** shrimp culture facilities in College Station and Galveston facilities in Texas. Considerable time and effort on the part of all project partners, their entity grant administration offices, and **BARD** was required to successfully complete this transfer of responsibilities, however in the end, a successful transfer was facilitated, albeit after much delay in the technical and scientific progress of the Year 2 tasks assigned to **TAMUS**.

During this period SIO expended considerable effort in project coordination and facilitation, but by the summer of 2015, a successful and relatively smooth transition of project

responsibilities from **TAMUS** to **AUBURN** was completed, and **BARD** thereafter allowed the participating institutions to draw down on their budgeted 2nd year funding.

YEAR 3 – Research Results

1) Partner Organization: University of Connecticut (UCONN)

Summary:

The University of Connecticut's (UCONN) Seaweed Marine Biotechnology Laboratory has been maintaining over 20 strains of *Gracilaria* and *Ulva* species collected from the US, Mexico, Portugal, China and Korea. Some of these strains are bloom forming species (*Ulva* spp.) or invasive species (*Gracilaria vermiculophylla*). These species grow fast, have a high tolerance to stresses and store nutrients in tissue at a high level. These characteristics make these strains suitable for the purpose of this project, providing sustainable biomass and a high quality product for incorporation into marine shrimp feeds. Among the strains developed at UCONN's Seaweed Marine Biotechnology Laboratory, fast growing strains have transferred to partner institutions. In 2014, 500 grams, fresh weight (FW) of *Gracilaria vermiculophylla* (G-NY-ST4) and 500 grams FW of *Ulva compressa* (U-CC-ST1) were transferred to UCSD-SIO for experimentation. UCONN also delivered *Ulva compressa* (U-CC-ST1) and *Gracilaria* (G-NY-ST4 and GV-KR-ST1) strains to Israel Oceanographic and Limnological Research Institute and The National Center for Mariculture (Haifa and Eilat) for experimentation in 2015. Finally, in 2016, UCONN transferred > 6 kg DW of *Ulva compressa* (U-CC-ST1) to Auburn University for experimentation for a constituent of shrimp feeds. UCONN has also conducted laboratory experiments, 1) to develop new culture media for land based *Gracilaria* cultivation, 2) to determine the growth and reproductive characteristics of the world largest bloom forming species, *Ulva prolifera,* 3) to compare the growth capacity and tissue composition of the non-native seaweed *Gracilaria vermiculophylla* in the Long Island Sound and the native *Gracilaria tikvahiae* and 4) to introduce *Ascophyllum nodosum* extract, called Acadian Marine Plant Extract Powder (AMPEP) to the *Gracilaria* cultures that may improve the desiccation tolerance in the spray cultivation systems.

As of the date of submission of this final project report, UCONN has published three (3) scientific papers and has submitted two (2) additional manuscripts to journals for consideration for future publication.

1. Development of new culture media for *Gracilaria* cultivation[4] (Kim J.K. and C. Yarish. 2014. Development of a sustainable land-based *Gracilaria* cultivation system. Algae 29: 217-225).

2. Characterization of the growth capability of the world largest bloom forming species, *Ulva prolifera*[5] [Zhang J., J.K. Kim, C. Yarish and P. He. 2016. The expansion of *Ulva prolifera* O.F. Müller macroalgal blooms in the Yellow Sea, PR China, through asexual reproduction. Marine Pollution Bulletin. 104:101-106)].

3. The effect of temperature on growth and tissue composition of the non-native seaweed *Gracilaria vermiculophylla* in the Long Island Sound compared to native *Gracilaria tikvahiae*[6]

<u>Task / Objective 1:</u> Collect and Screen red and green seaweed candidates from several genera including *Gracilaria* and *Ulva* for temperature tolerance and growth under spray cultivation conditions.

***Gracilaria:* (red marine alga)**

During previous projects supported by the US EPA Long Island Sound Futures Fund (NFWF/Legacy Grant Project ID: 1401.10.024266), CT Sea Grant (R/A 38, NA10OAR4170095), Woods Hole Sea Grant College Programs (NA10OAR4170083) and the U.S. Department of Energy's NETL Program (FOA #0000015), over 10 strains of the *Gracilaria tikvahiae and G. vermiculophylla* species from CT, RI, MA and NY were isolated and have been maintained at UCONN's Seaweed Marine Biotechnology Laboratory. During the project period, we isolated an additional strain of *G. vermiculophylla* from Qingdao,

[4] This study was also supported by the U.S. EPA Long Island Sound Study's Long Island Sound Futures Fund, New York State Attorney General's Bronx River Watershed Initiative Grant Program, National Fish and Wildlife Foundation (NFWF/Legacy Grant Project IDs: 1401.10.024266 and 8012.08.030370) and Connecticut Sea Grant College Program (R/A-38).

[5] This study was also supported by a grant to P. He and J. Zhang from the Ocean Public Welfare Scientific Research Project, China (201205010).

[6] This study was also supported by the U.S. EPA Long Island Sound Study's Long Island Sound Futures Fund, New York State Attorney General's Bronx River Watershed Initiative Grant Program, National Fish and Wildlife Foundation (NFWF/Legacy Grant Project IDs: 1401.10.024266 and 8012.08.030370) and Connecticut Sea Grant College Program (R/A-38).

China. Species of all strains was determined by DNA sequencing using ribosomal small-subunit 18s RNA. Additionally, *G. vermiculophylla*, collected from east coast of Korea, which is a haplotype of all invasive strains of this alga in Europe and North America, was also transferred from Prof. S.M. Boo's laboratory (Chungnam University, Korea) to UCONN's Seaweed marine Biotechnology Laboratory. An additional *Gracilaria* species was also isolated using samples collected by Dr. Charles Yarish during his sampling trip to La Paz, Baja California Sur, Mexico (Oct. 18, 2014 – Oct. 19, 2014). The *Gracilaria* was collected from Pichilingue, La Concha and Las Pacas, Baja California Sur, Mexico. This *Gracilaria* species was identified as *G. parvispora* by DNA sequencing using ribosomal small-subunit 18s RNA. This species is an invasive species to Mexico (native to Hawaii, USA).

AT UCONN, we have established and propagated vegetative cultures of male and female gametophytes, and tetrasporophytes, which was isolated from spores. We also established cultures initiated from field collected plants through vegetative propagation. In both cases, clean plants in good condition were collected from the field and cleaned by gently wiping with sterile cotton balls. Vegetative branches were dragged through seaweed agar to remove epiphytes. For vegetative cloning, the excised vegetative tips were transferred to von Stosch's enriched seawater (VSE) in 50 mm sterile Petri dishes and maintained at 20°C, 20 μmol m^{-2} s^{-1} light and day neutral (12:12, L:D) conditions (Ott, 1965). Tetrasporic and cystocarpic branches were transferred to sterile culture dishes containing VSE and were maintained at 20°C, 10 μmol m^{-2} s^{-1} light and day neutral conditions until spores were released and begin to germinate. Individual germlings were transferred to VSE in 50 mm sterile culture dishes and maintained at 20°C, 20 μmol m^{-2} s^{-1} light and day neutral conditions.

After we selected a fast growing strain of *Gracilaria vermiculophylla* (GV-NY-ST1), we began to mass culture it from Aug., 2013 at UCONN and at our satellite facility at BRASTEC in volumes ranging from 1-200L. On March 11, 2014, UCSD received approximately, 500 grams of *Gracilaria vermiculophylla* from UCONN for experimentation.

***Ulva*: (green marine alga)**

During the current project period, we isolated two new strains of *Ulva* from Bridgeport, CT and Jamaica Bay, Queens, NY. Healthy thalli were transported to the laboratory on ice within 24 h. Disks from the marginal section of the thalli were cleaned by gently wiping with sterile

cotton balls. The disks were transferred to von Stosch's enriched seawater (VSE) in 50 mm sterile Petri dishes and maintained at 20°C, 20 μmol m^{-2} s^{-1} light and day neutral (12:12, L:D) conditions (Ott, 1965). By the next day, zoospores or parthenogametes were released from the disks, and a few of these propagules were placed on slides (10mm X 10 mm) in sterile Petri dishes for the development of new thalli. The species were molecularly identified as *U. compressa* and *U. laetevirens*, respectively, using tufA and ITS genes. Additionally, *U. prolifera*, and *U. linza* (collected from Rudong Sea, China) were transferred from Prof. P. He's laboratory (Shanghai Ocean University, China) to UCONN's laboratory. The *U. prolifera* is the same haplotype of the world's largest macroalgal bloom forming strain from The Yellow Sea, China. Additionally, another *Ulva* species was isolated using samples collected by Dr. Charles Yarish during his sampling trip to La Paz, Baja California Sur, Mexico (Oct. 18, 2014 – Oct. 19, 2014). The *Ulva* was originally collected from The Melcone Beach (the main beach) of La Paz. It was initially identified as *U. ohnoi*. Currently, UCONN is maintaining these five *Ulva* species in its culture collection (Fig. 1).

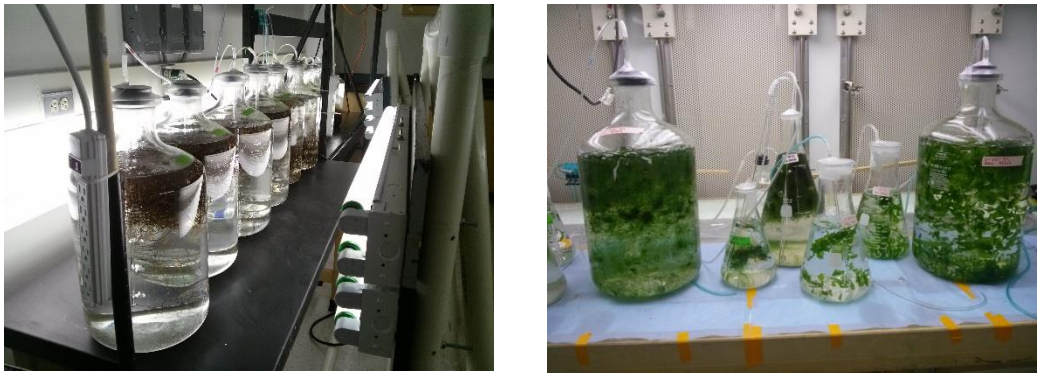

Figure 1. Clonal cultures of *Gracilaria* (left) and *Ulva* at UCONN Stamford Laboratory.

It is important to note that some of strains developed in this study are bloom forming species (*Ulva* spp.) or invasive species (*Gracilaria vermiculophylla*). These species grow fast, have a broad tolerance to stress (temperature, light and desiccation, etc.) and store nutrients in tissue at very high levels. These characteristics will make our strains sustainable for the purpose of this project, providing biomass and a high quality constituent for animal feeds. Since these species are invasive or bloom forming species in the coastal oceans, the biosecurity in the culture systems are extremely important, which our land based (spray) culture systems can afford. In our laboratory studies, we have determined the physiological characteristics of these species and compared the growth capacities in between native and invasive species, to

suggest the most suitable species for the purpose of this project. In addition, we also developed a novel land based tank culture technique, which is economically sustainable.

Material Transfer from UCONN to BARD Partner Institutions

- UCONN has increased biomass of *Ulva compressa* and *Gracilaria vermiculophylla* at our UCONN and Bridgeport Regional Aquaculture Science, Technology Education Center (BRASTEC) labs in volumes ranging from 1-200L (Figure 2). The biomass has been made available for experimentation at UCONN, as well as our partner institutions.
- On March 11, 2014, UCSD received approximately, 500 grams of *Gracilaria vermiculophylla* from UCONN for experimentation.
- On Dec. 8th 2014, UCONN sent approximately 500 grams, fresh weight of *Ulva compressa* (strain ID: U-CC-ST1) to UCSD-SIO for experimentation.
- During Dr. Yarish's UCONN sponsored trip to Israel (Jan. 7, - Jan. 9, 2015), Dr. Yarish delivered UCONN's *Ulva compressa* (U-CC-ST1) and *Gracilaria vermiculophylla* (G-NY-ST4 and GV-KR-ST1) strains to Israel Oceanographic and Limnological Research Institute and The National Center for Mariculture (Haifa and Eilat) for experimentation.
- On March 8, 2016, > 6 kg DW of *Ulva compressa* (U-CC-ST1) cultivated and dried at UCONN were shipped to Auburn University for experimentation for a constituent of shrimp feeds.

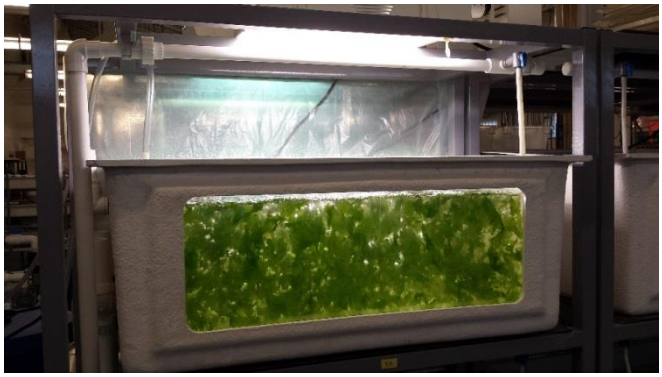

Figure 2. *Ulva compressa* growing in 200 L tank at Bridgeport Regional Aquaculture Science and Technology Education Center (BRASTEC)

Table 1. Nutrient composition in each media

	VSE	JS	MG
Nitrogen	500 μM	500 μM (41% NH_3 and 59% NO_3)	500 μM (15% NH_3 and 85% Urea)
Phosphorus	30 μM	39 μM	34 μM
Iron	1 μM	0.6 μM	0.8 μM
Manganese	10 μM	0.3 μM	0.3 μM
EDTA	10 μM	not specified by maker	not specified by maker
Vitamins	Yes	-	-

The commonly used nutrient medium for cultivation of marine red macroalgal is the von Stosch Seawater enrichment (VSE) media (Ott, 1965). VSE contains several important nutrients including nitrate, phosphate, iron, manganese, EDTA and vitamins. However, VSE may not be applicable for the commercial or large scale cultivation due to its high costs for material and preparation. We have evaluated two potential culture media for *Gracilaria* cultivation using commercially available fertilizers (Table 1). Since vitamins are included in VSE, five media conditions were utilized, 1) VSE, 2) JS, 3) JSV, 4) MG and 5) MGV. Total nitrogen and phosphorus concentrations in each medium were adjusted as same those in VSE (500 μM and 30-39 μM, respectively; Table 1). However, the nitrogen sources were different in each media (VSE, 100% NO_3, JS, 41% NH_3 and 59% NO_3 and MG, 15% NH_3 and 85% Urea). *Gracilaria* was cultivated at 100 μmol m^{-2} s^{-1}, 20°C and 12:12 L:D of photoperiod. The stocking density was 2 g L^{-1}. At one-week intervals for four weeks, all of the biomass in each flask was weighed (fresh weight; FW) and samples were taken for tissue analyses. For the analysis of tissue total N content, samples were dried at 60 °C before being ground. The powder was analyzed using a Perkin Elmer 2400 series II CHNS/O elemental analyzer.

The *Gracilaria* cultivated in the medium with JS fertilizer grew as well as it did in VSE. The growth rates of *Gracilaria* in VSE, JS and JSV were 6.8-11.0%, 5.8-11.8% and 6.2-10.5% d^{-1}, respectively. The growth rates using MG (5.5-8.1% d^{-1}) and MGV (4.5-7.4% d^{-1}) were lower than those in other conditions (Fig. 3). Tissue nitrogen contents were also higher at VSE, JS and JSV than at MG and MGV (Fig. 4). Vitamins did not show any significant effect on the growth of this species, at least over the 7 days' growth between culture medium change.
When the price of each media was compared (Table 2), JS proved to be the least expensive ($0.01 per m^3 of medium) than VSE ($1.62). Although the costs for vitamins were removed from VSE, the cost for CF1 media is still 2% of the cost for VSE without vitamins. This

result suggests a potential uses of commercially available fertilizer in seaweed nursery systems.

Figure 3. Comparison of growth rates of *Gracilaria tikvahiae* grown at different fertilizers. VSE: von Stosch enrichment media; JS: Jack's Special™ only (JS; N:P:K, 21:8:18); JSV: JS with vitamins; MG: Miracle-Gro® only (MG; N:P:K, 24:8:16); MGV: MG with vitamins.

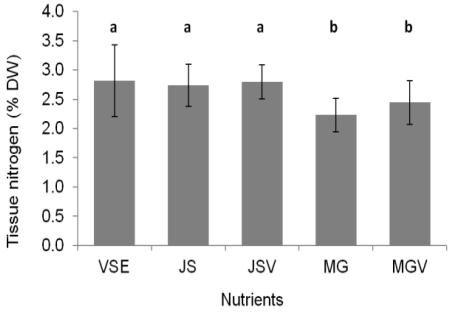

Figure 4. Comparison of tissue nitrogen (N) of *Gracilaria tikvahiae* grown at different fertilizers. VSE: von Stosch enrichment media; JS: Jack's Special™ only (JS; N:P:K, 21:8:18); JSV: JS with vitamins; MG: Miracle-Gro® only (MG; N:P:K, 24:8:16); MGV: MG with vitamins.

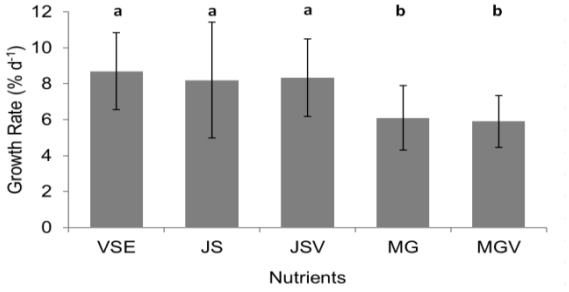

Table 2. Price comparisons of different culture media

Nutrients	Price per m^3 of culture medium
VSE	$1.623
JS	$0.010
JSV	$1.114
MG	$0.012
MGV	$1.116

Ulva prolifera has been identified as the dominant species in the world largest macroalgal blooms in the Yellow Sea of China. Some *Ulva* species are known to have polarized growth when the thalli were cut into small pieces. Müller-Stoll (1952) first observed that in *U. compressa,* new rhizoids were formed at the basal cut of the thalli while the upper cut surfaces showed the non-rhizoidal cells, called 'papillae'. Eaton et al. (1966) and Moss & Marsland (1976) also found a similar phenomenon in *U. intestinalis*. The objectives of this study were to identify the different developmental strategies in *U. prolifera*, to determine environmental conditions forming the polarized growth.

Fresh green tubular thalli without branches were selected and cut into 5 mm segments in all experiments. The segments were transferred into Pyrex Petri dishes (7 cm in diameter) containing 5 mL VSE medium. The experiments were performed at irradiances of 50, 75 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperatures from 10 to 25°C in 5°C for two weeks.

We found that one 5 mm segment of *U. prolifera* could form 25 new thalli on average, and each thallus grew, matured, and released spores in four weeks (Fig. 5). Among *Ulva* species, this growth strategy was reported only in *U. prolifera.* Previous studies also showed the polarized growth patterns in *Ulva* species, however, other *Ulva* species including *U. compressa,* formed only one or two new thallus from the upper cut surfaces. Another interesting finding in the present study is that the polarized growth was mostly observed at high temperature (≥ 20 °C) and low light (≤ 75 μmol·m^{-2}·s^{-1}) conditions. Otherwise, most segments completely released germ cells. This growth and reproductive patterns in *U. prolifera* segments can explain the outstanding growth of this alga, and forming the world largest macroalgal blooms in China.

Figure. 5. Developmental process of *U. prolifera* polarized segments. **a**, *U. prolifera* segments; **b**, polarized growth of *U. prolifera* segments; **c-d**, new blades from the upper cut surface of the *Ulva* segments; **e-f**, rhizoids generated from the basal cut surface of the *Ulva* segments; **g-i**, continuous growth of one individual *Ulva* segment; **j-l**, new *Ulva* younger thalli generated from the germ cells of *Ulva* grown segments.

In the past two decades, the red alga *Gracilaria vermiculophylla*, a species native to the waters of Korea and Japan, has invaded marine coastal areas of Europe and the Americas,

thriving in conditions that differ from those of its native habitat. In recent years, *G. vermiculophylla* has been discovered in the Long Island Sound (LIS) growing alongside the native congener *Gracilaria tikvahiae*. The goal of this study was to determine whether the *G. vermiculophylla* strain growing in LIS exhibits phenotypic plasticity, and whether physiological differences can explain the success of the invasive species. Two strains of *Gracilaria vermiculophylla* (isolated in Korea, GV-KR-ST1 (courtesy of S.M. Boo, Daejeon, Korea) and LIS, G-NY-ST4) and a strain of LIS native *Gracilaria tikvahiae* (G-RI-G1) were grown in von Stosch's enriched (VSE) medium for four weeks under temperatures ranging from 20° C to 34° C using a temperature gradient table, while all other environmental conditions (100 μmol m^{-2} s^{-1}, 12:12 L:D photoperiod and 30 ppt salinity) remained constant. At the end of each week, the wet weight of each sample was recorded. Thalli were reduced to the original stocking density of 1 g L^{-1}, excess biomass was preserved for tissue carbon and nitrogen analysis, and water samples were collected.

Growth Rate

Overall, the LIS strain of *Gracilaria vermiculophylla* appeared to grow at rates more similar to *G. tikvahiae* than to those of the Korean strain. At each of the tested temperatures, the Korean strain of *G. vermiculophylla* outperformed the LIS strain and *G. tikvahiae*. During the first week, at 24 °C and 29 °C the Korean strain of *G. vermiculophylla* grew at rates of 22.9% and 23.2%, respectively. By week four at 24 °C and 29 °C, the Korean strain grew at only at 10.0% and 9.4%, respectively. Under the same conditions in the first week the LIS strain grew at 6.2% and 5.7%, and *G. tikvahiae* grew at 12.0% and 9.4%. For 20, 24, and 29 °C treatments, all strains displayed a general decline in growth rate over time (Figure 6-8). However, at 34 °C, the growth rate of both *G. vermiculophylla* strains slightly increased. *Gracilaria tikvahiae* failed to survive at 34 °C. It lost its' pigment and became bleached within the first week of exposure and was removed from growth experimentation. By the end of the second week, one replicate of *G. tikvahiae* at 29 °C failed to survive and it too was removed from experimentation (Figure 9).

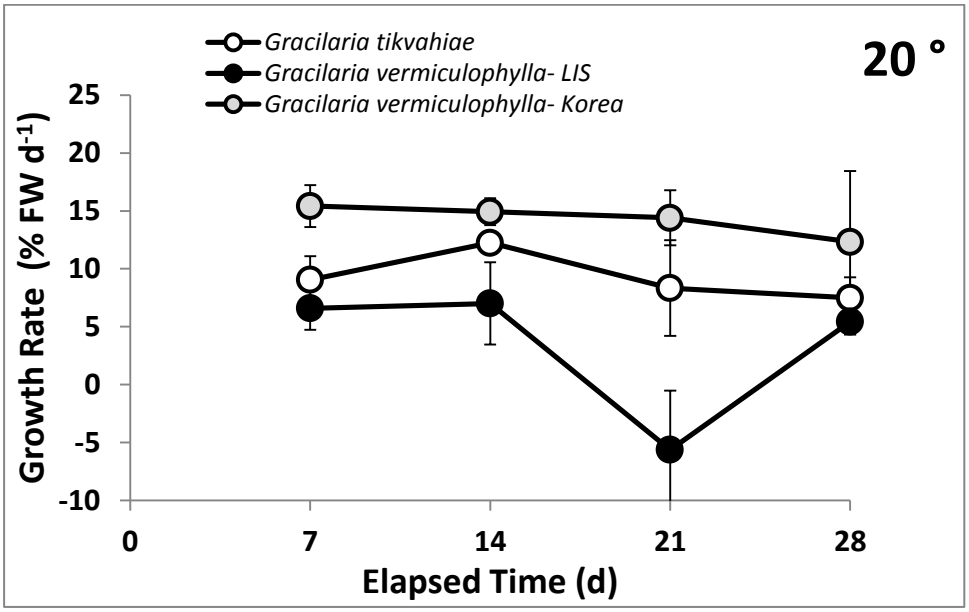

Figure 6. Average weekly growth rate measure in percent growth per day of each *Gracilaria* strain under the 20 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

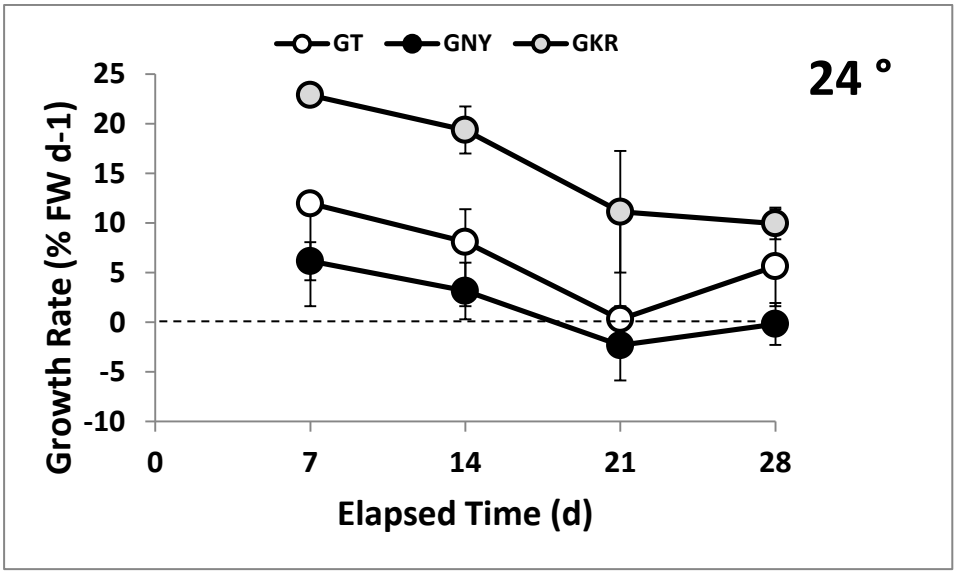

Figure 7. Average weekly growth rate measure in percent growth per day of each of *Gracilaria* strain under the 24 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

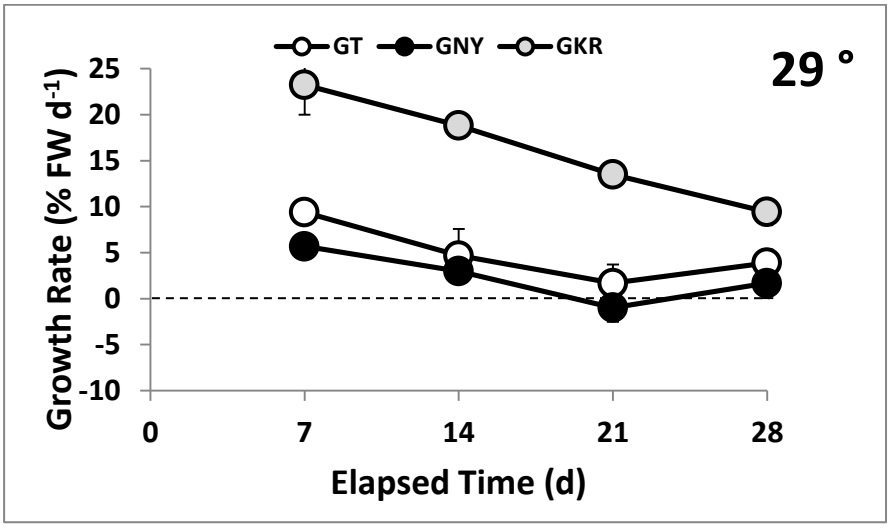

Figure 8. Average weekly growth rate measure in percent growth per day of each *Gracilaria* strain under the 29 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

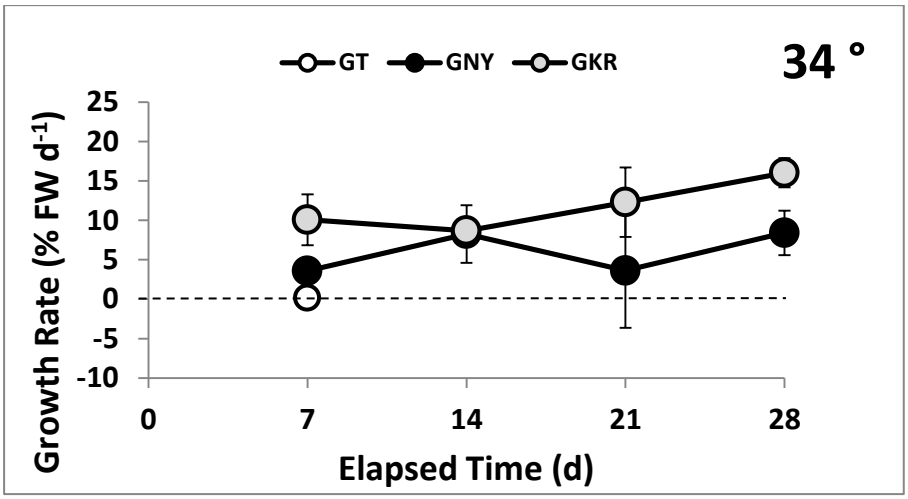

Figure 9. Average weekly growth rate measure in percent growth per day of each *Gracilaria* strain under the 34 °C temperature treatment. Error bars are not present for plots with minor standard deviation. *Gracilaria tikvahiae* is not displayed past week one as no replicates survived under this condition.

Tissue Nitrogen

For each temperature treatment, the LIS strain of *Gracilaria vermiculophylla* consistently concentrated more N in its tissue than the Korean strain and the LIS native *G. tikvahiae*. There was one exception at 34 °C treatment in week 4 where the Korean strain had a higher average concentration (Figure 10). The LIS strain exhibited nitrogen levels ca. 4-5% N (DW), whereas the Korean strain and *G. tikvahiae* produced tissue with 2-3% N, DW (Figure

10-13). For all three strains, tissue N remained relatively constant over the four-week period. For both the LIS and Korean strains of *Gracilaria vermiculophylla* tissue N appears to be influenced by temperature; as temperature increased, tissue N tended to decrease. However, this trend was not apparent with *Gracilaria tikvahiae*.

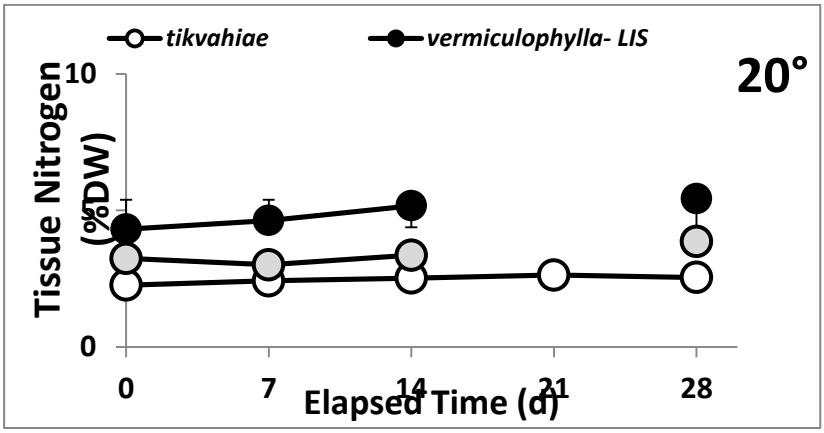

Figure 10. Average percent tissue nitrogen in each dried sample after each of four weeks for each *Gracilaria* strain under the 20 °C temperature treatment. The week three measurements for *G. vermiculophylla* strains were not made. Error bars are not present for plots with minor standard deviation.

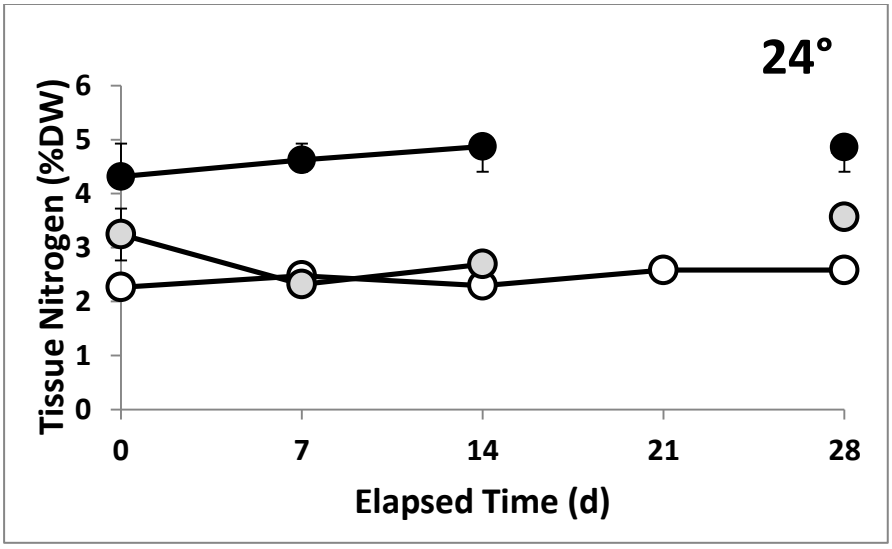

Figure 11. Average percent tissue nitrogen within each dried sample after each of four weeks for each *Gracilaria* strain under the 24 °C temperature treatment. The week three measurements for *G. vermiculophylla* strains were not made. Error bars are not present for plots with minor standard deviation.

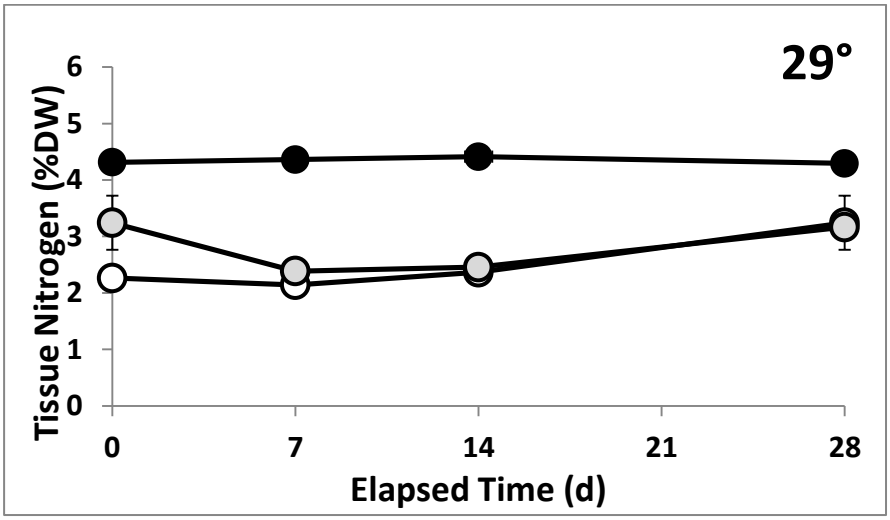

Figure 12. Average percent tissue nitrogen within each dried sample after each of four weeks for each *Gracilaria* strain under the 29 °C temperature treatment. Error bars are not present for plots with minor standard deviation.

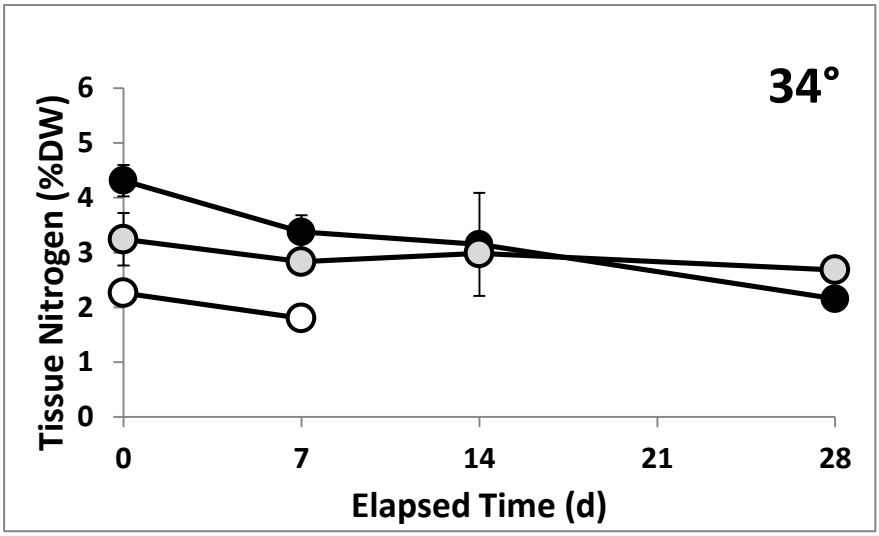

Figure 13. Average percent tissue nitrogen within each dried sample after each of four weeks for each *Gracilaria* strain under the 34 °C temperature treatment. *Gracilaria tikvahiae* is not displayed after week 1 as no replicates survived under this condition and was therefore removed from the study.

Statistics

An analysis of variance (ANOVA) was conducted on results of growth rate and tissue nitrogen for week four of the growth period and showed significant differences among strains for each temperature treatment with the exception of growth rate at the 20 °C treatment. For growth rate, the *Gracilaria vermiculophylla* strain from Korea always had faster growth rates than rates of the other strains at 24, 29, and 34 °C temperature treatments. The difference between the growth rates of *G. vermiculophylla* LIS strain and *G. tikvahiae* at 29 °C was not

significant (Figure 14). The difference between tissue N concentrations of each strain was significant for every temperature with the exception of the 29 °C treatment. In the 29 °C during week 4, no significant difference between the Korean strain of *G. vermiculophylla* and *G. tikvahiae* were found (Figure 15).

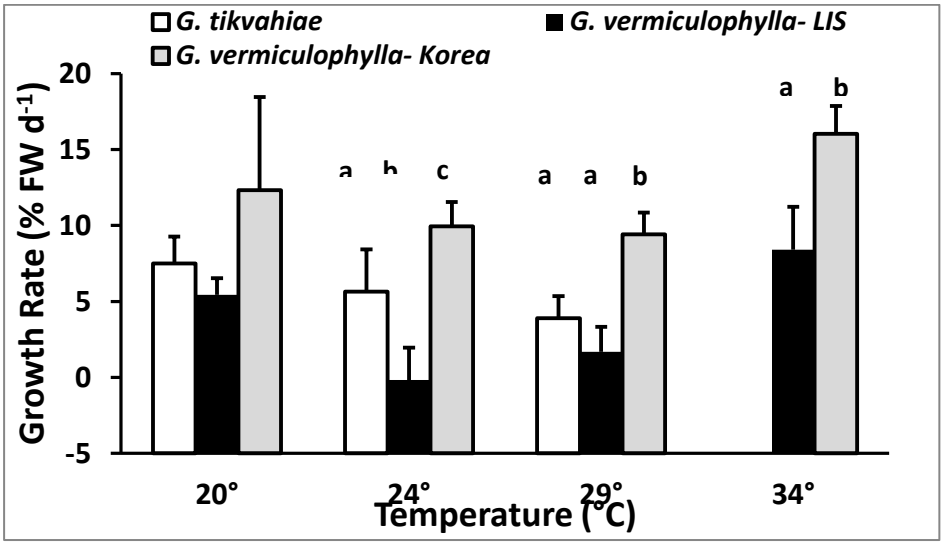

Figure 14. Average growth rate during week 4 in percent growth per day for each *Gracilaria* strain under temperature conditions 20, 24, 29, and 34 °C. Letters above bars denote results of a Fisher LSD Test comparing the strains within each temperature treatment after an ANOVA revealed significant difference among strains for 24, 29, and 34 °C temperature treatments.

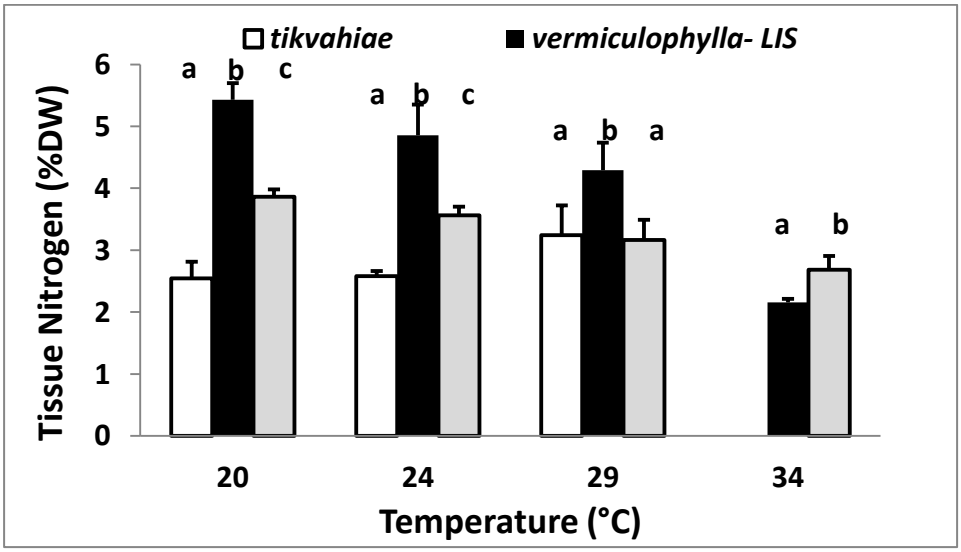

Figure 15. Average percent tissue nitrogen during week 4 for each *Gracilaria* strain under temperature conditions 20, 24, 29, and 34 °C. Letters above bars denote results of a Fisher LSD Test comparing the strains within each temperature treatment after an ANOVA revealed significant difference among strains.

1.2. Effects of desiccation in *Gracilaria,* simulating the spray cultivation systems, treated with *Ascophyllum nodosum* extract (AMPEP)[7]

The brown fucoid algae, *Ascophyllum nodosum,* commonly known as knotted wrack or rockweed, exists in the eulittoral zone in the North Atlantic. It is one of the most researched seaweeds in the northern hemisphere (Khan et al. 2011). An extract of *Ascophyllum nodosum* (Acadian marine plant extract powder – AMPEP) has been the basis of numerous studies, most recently with the economically important red macroalga, *Kappaphycus alvarezii* (Loureiro et al. 2010, Hurtado et al. 2009). *A. nodosum* extract **(**AMPEP) has proven to increase the temperature tolerance, otherwise considered lethal, as compared to control samples (Loureiro et al. 2010). AMPEP has also been shown to reduce epiphytes and increase disease resistance (e.g. "ice-ice" and "goosebumps") at concentrations of just 15 and 20 grams L^{-1} (Loureiro et al. 2010). Effects on terrestrial organisms have proven similar results. Cucumber plants exposed to 1% *Ascophyllum* derived solution applied twice daily for 10 days increased its resistance to a wide range of pathogens and parasites, while displaying increased biomass over the control samples (Jayaraj et al. 2011).

The objective of this study was to determine if *A. nodosum* extract **(**AMPEP) increases the growth capacity of *Gracilaria vermiculophylla* and increases its tolerance to desiccation stress. Experimentation utilized a tide-simulating apparatus (Kim and Yarish 2010). This device allows marine macroalgae cultures to endure periods of submergence and exposure in regular intervals simulating tidal exposure. The flexibility in adjusting tide times for the tide-simulating apparatus (1 of table for treatment and another for a control with each table containing 18 individual replicate cylinders), gives an opportunity to explore emersion stress at regulated intervals, as well as static conditions (control or no emersion).

Gracilaria vermiculophylla (GV-KR-ST1) was cultivated in 2.5 L cylinders containing six different concentrations of AMPEP (0, 10, 20, 30, 40 and 50 mg /L; n=3) using the tide simulating apparatus. Of the 36 cylinders in total, 18 cylinders of *G. vermiculophylla* remained submerged while the *Gracilaria* in the other 18 experienced emersion stress with a

[7] This study was also supported by the U.S. EPA Long Island Sound Study's Long Island Sound Futures Fund, New York State Attorney General's Bronx River Watershed Initiative Grant Program, National Fish and Wildlife Foundation (NFWF/Legacy Grant Project IDs: 1401.10.024266 and 8012.08.030370) and Connecticut Sea Grant College Program (R/A-38).

15 minute / 1 minute emersion/submergence cycle. A fifteen-minute exposure caused approximately 10% water loss in *Gracilaria*.

Emersion stress significantly affected the growth of *Gracilaria vermiculophylla*. When *G. vermiculophylla* didn't experience emersion stress, interestingly, addition of AMPEP reduced the growth rate of this alga. However, when *G. vermiculophylla* was exposed to air on a regular basis, a low concentration of AMPEP addition (10 mg L^{-1}) increased the growth capacity (Figure 16).

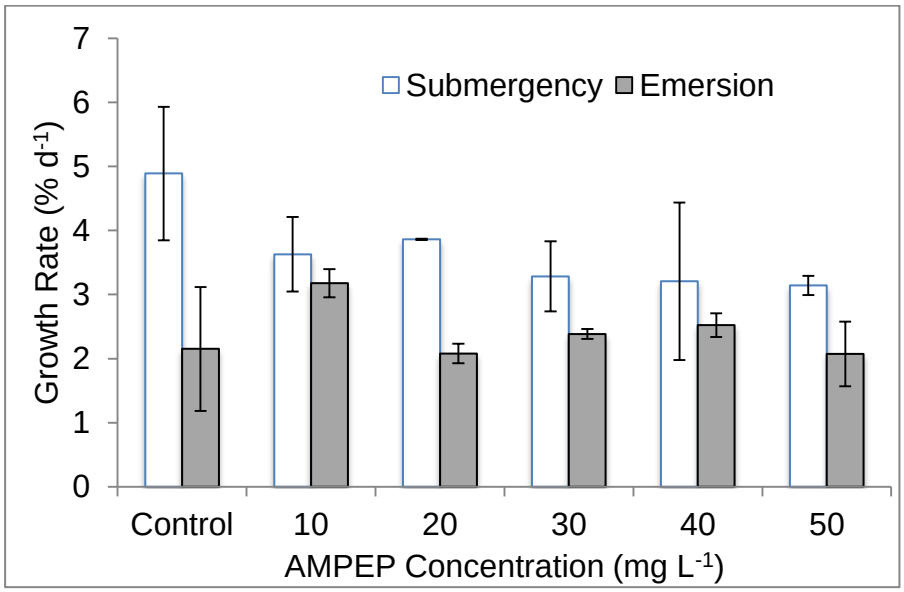

Figure 16. Growth rates of *Gracilaria vermiculophylla* cultivated at six different concentrations of *Ascophyllum* extract, AMPEP (0, 10, 20, 30, 40 and 50 mg /L; n=3) with or without emersion stress. Samples with emersion stress was exposed to air with emersion stress with a 15 min / 1 min emersion/submergence cycle. Error bars represent standard deviation.

Publications Resulting from this work (UCONN):

Kim, J.K. and Yarish C. 2014. Development of a sustainable land-based *Gracilaria* cultivation system. Algae. 29: 217-225.

Zhang, J., Kim J.K., Yarish C. and He P. 2016. The expansion of *Ulva prolifera* O.F. Müller macroalgal blooms in the Yellow Sea, PR China, through asexual reproduction. Marine Pollution Bulletin. 104:101-106.

Kim, J.K., Yarish C. and Pereira R. 2016. Tolerances to Hypo-osmotic and Temperature Stresses in native and invasive *Gracilaria* species. Phycologia. 55: 257-264.

Kim, J.K., Yarish C. et al. (submitted) Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services. Algae.

Gorman, L., Kraemer G.P., Yarish C., Boo S.M. and Kim J.K. (submitted) The effects of temperature on the growth and nitrogen content of *Gracilaria vermiculophylla* and *Gracilaria tikvahiae* from LIS, USA. Algae.

Presentations (UCONN):

Gorman L., Kim J.K, Yarish C. and Kraemer G. 2015. The effect of temperature on growth of non-native seaweed species *Gracilaria vermiculophylla* in the Long Island Sound as compared to native *Gracilaria tikvahiae.* SUNY Undergraduate Research Conference. The College at Brockport, State University of New York. April 10, 2015.

Other Activities (UCONN):

- Drs. Yarish and Kim visited UCSD / Scripps Institution of Oceanography as part a bilateral program between NOAA and The *National Fisheries Research and Development Institute* (*NFRDI*) of Korea on Mar. 23rd, 2015. Drs. Yarish and Kim discussed seaweed cultivation systems and experimental design for the project with project PIs, Drs. B. Greg Mitchell and Dominick Mendola, as well as the post-doctoral associate, Dr. Wilson Mendoza of UCSD-SIO.

- Dr. Yarish also visit IOLR and NCM, Haifa and Eilat, Israel, with the support of The UCONN Office of Global Affairs (Jan. 7, - Jan. 9, 2015). During this trip, Dr. Yarish delivered UCONN's *Ulva compressa* (U-CC-ST1) and *Gracilaria* vermiculophylla (G-NY-ST4 and GV-KR-ST1) strains to Israel Oceanographic and Limnological Research Institute and The National Center for Mariculture (Haifa and Eilat) and made presentations at our partner institutions.

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2) **Partner Organization:** UCSD, Scripps Institution of Oceanography (UCSD-SIO)

Task / Objectives:

Descriptive Title of Work: Laboratory Growth performance of *Ulva lactuca* for three different re-circulating seawater spray system designs.

1. SUMMARY of Year 3 Methods and Results

Economical cultivation of large-scale, land-based seaweed culture systems is desired to provide a sustainable source of marine plant protein for formulating renewable aquaculture feeds, for agar production, and for provision of biomass for generating renewable energy. We chose to investigate water-sparing spray culture of the green marine seaweed *Ulva lactuca* (*Ulva*) in a laboratory setting. We conceived three different structural designs for support of the growing biomass of the seaweed as part of a recirculating seawater spray system. The three support designs were: (1) bag-pocket vertical design (BPVD); (2) multi-level horizontal (MLHD); and (3) slant-horizontal design (SHD). Growth rates of the Ulva seaweed using these cultivation methods were compared with fully submerged *Ulva* cultures, e.g. the controls (SUB). Replicate spray culture growth experiments were run for approximate 2 weeks periods, and repeated in serial order throughout the 3rd year research period, with breaks in between experiments to analyze data obtained. Results were treated statistically to test the interaction effect of cultivation designs using a four-level single factorial randomized block experimental framework ($p<0.05$). In another statistical treatment, we used a four-level two factor experimental design to test for growth performance and protein content of *Ulva* and any interaction effects of cultivation designs and differing concentrations of Jack's

Special culture Media (JSM), a commercial plant fertilizer. Results demonstrated that the tested spray system designs showed promising results for improving the growth rate of Ulva using the MHLD design and 2 x JSM, indicating that the MHLD design would be the best culture configuration to use for follow-on, larger-scale testing in an outdoor pilot-scale greenhouse *Ulva* cultivation spray system.

2. Materials and Methods

Seaweed culture and stocking density

A mini-spray culture experimental array, in a randomized framework (Figure 1), was employed to perform testing on the UCONN-isolated strains of *U. lactuca* for physiological tolerance to culture protocol factors of light and nutrients using von Jack's Special Medial. Three replicate polyethylene containers were used per test condition.

Dry Biomass

*Dry biomass = (Wtf * (HWti/Wti)/A)/d (g/m2/day)*

Wtf =Final dry weight

HWti/Wts = wet biomass correction factor: (highest wet biomass weight / initial mean wet weight of sample for each design grouping). Initial wet weight differences for groupings = ± 0.01 g

A= m2 (0.004 m2, normalized area of sample; 6.35 cm^2)

d = number of cultivation days

% Growth rate

% Growth rate = $[(Wt/Wo)^{1/t}-1*100]$

Growth rate (as %GR/day) was calculated using the difference of the final and initial weight divided by the number of days in the culture and expressed as % $[(Wt/Wo)^{1/t}-1*100]$ (Yong et al., 2013). The relative growth rate per day (RGR/day) was computed using the formula (ln Wt-lnWo/t) * 100. (Glen and Doty 1992). Each experimental design-unit contain twelve pieces of ~1 g of pre-weighed seaweed seedlings (total trial = 12). The high number of trials in each replicate unit minimizes the effect of lost branches during seedling preparation and during harvest. Three replicates for each design cultivation unit were used, incorporating three spray system designs and one submerged system design: Multi-level horizontal design

(MHLD), (Bag-String Vertical design (BSVD), Netted-Vertical Design (NVD) and S(submerged System) : See photo for graphical representation of each design (Figure 2).

Nutrient Analysis:

https://scripps.ucsd.edu/ships/shipboard-technical-support/odf/documentation/nutrient-analysis

Equipment and Techniques

Nutrient analyses (phosphate, silicate, nitrate plus nitrite, and nitrite) are performed on aSeal Analytical continuous-flow AutoAnalyzer 3 (AA3). After each run, the charts are reviewed for any problems and final concentrations (in micromoles per liter) are calculated using SEAL Analytical AACE 6.07 software.

The analytical methods used are described by Gordon et al. [Gord92], Hager et al. [Hage68] and Atlas et al. [Atla71]. The details of modification of analytical methods used for this cruise are also compatible with the methods described in the nutrient section of the GO-SHIP repeat hydrography manual [Hyde10].

Nitrate/Nitrite Analysis

A modification of the Armstrong et al. [Arms67] procedure is used for the analysis of nitrate and nitrite. For nitrate analysis, a seawater sample is passed through a cadmium column where the nitrate is reduced to nitrite. This nitrite is then diazotized with sulfanilamide and coupled with N-(1-naphthyl)-ethylenediamine to form a red dye. The sample is then passed through a 10mm flowcell and absorbance measured at 540nm. The procedure is the same for the nitrite analysis but without the cadmium column.

Phosphate Analysis

Ortho-Phosphate is analyzed using a modification of the Bernhardt and Wilhelms [Bern67] method. Acidified ammonium molybdate is added to a seawater sample to produce phosphomolybdic acid, which is then reduced to phosphomolybdous acid (a blue compound) following the addition of dihydrazine sulfate. The sample is passed through a 10mm flowcell and absorbance measured at 820nm.

Sampling Requirements and Preservation

Freezing is the preferred method of preservation. 20-30 mL of sample are required for analysis. Polypropylene screw-capped centrifuge tubes are recommended. Tubes should be sterile or washed thoroughly with 10% HCl and rinsed with sample at least 3 times prior to filling. Leave a headspace for expansion during freezing. Samples are thawed overnight at ~1.7 C and brought to room temperature prior to analysis. The centrifuge tubes fit directly onto the sampler.

Data collection and processing

Data collection and processing is done with the software (ACCE ver 6.07) provided with the instrument from Seal Analytical. After each run, the charts are reviewed for any problems during the run, any blank is subtracted, and final concentrations are calculated, based on a linear curve fit. Next, a text file is created. This is reviewed for possible problems and then converted to an output file with only sample identifiers and nutrient concentrations that can be merged with other bottle data.

Standards and Glassware calibration

Primary standards for silicate (Na2 SiF6), nitrate (KNO3), nitrite (NaNO2), and phosphate (KH2 PO4) are obtained from Johnson Matthey Chemical Co. and/or Fisher Scientific. The supplier reports purities of >98%, 99.999%, 97%, and 99.999 respectively.

All glass volumetric flasks and pipettes are gravimetrically calibrated. The primary standards are dried and weighed out to 0.1 mg. When primary standards are made, the flask volume at 20°C, the weight of the powder, and the temperature of the solution are used to buoyancy correct the weight, calculate the exact concentration of the solution, and determine how much of the primary is needed for the desired concentrations of secondary standard. New standards are compared to the old before use.

All the reagent solutions, primary and secondary standards are made with fresh distilled deionized water (DIW). Standardizations are performed at the beginning of each group of samples.

Kinetics of Nutrient Uptake

The steady state kinetics of nutrient uptake and seaweed growth is successfully used in the course of alga cultivation (Silkin et al., 1992 ; Silkin et al., 2007) on the basis of the

regulation of uptake process is the dependence of the uptake rate of the nutrient concentration in the medium that can be expressed by the equation of Michaelis-Menten:

$\upsilon = \upsilon_{max}$ x C/K+C , where C is the nutrient concentration in the medium; υ and υ_{max} is the specific and maximum specific uptake rate; and K is the half saturation constant , equal to the nutrient concentration, when $\upsilon = \upsilon max/2$, µmol.

Spectrophotometric Pigment measurement and calculation

Pigments were extracted from seaweeds using mortar and pestle in 90% acetone. Spectrum of extracted pigment were determined by a Varian Spectrophotometer. Chl a, chl b and carotene were calculated as follows:

Chl a (mg g-1) = [(11.75× A662) - (7.340× A645)] × V(ml) / mg seaweed tissue x1000
Chl b (mg g-1) = [(18.61 × A645) - (3.960 × A662)] × V(ml) / mg seaweed tissue x1000
Total Chl = Chl a + Chl b
*Carotenoid (mg/g) = 1000*A480 -(2.270 × chla) – (81.4 × chlb)/227*

Statistics

Normality of data were tested using Shapiro-Wilkinson test and Brown-Forsythe for variance equality test. The effects of the culture conditions and JSM concentrations on the growth rate were verified through one-way and two-way ANOVA. This was followed by Holm-Sidak method (significance level of 0.05) for the pairwise multiple comparison.

3. Results

The MHLD showed the highest mean daily biomass gain (~16g/m2/day) among the three spray system platform with the 2X JSM concentration (Figure 3). The growth rate of *U.lactuca* in MHLD platform with 2X JSM, exhibit a maximum of ~9%/day. With 80% water content, the estimated ash-free dry weight of *Ulva* on MHLD platform reached 13.18 g/m2/day, which is half the *Ulva* AFDW on the SUB platform.

Cellular uptake rates of dissolved nutrients were estimated using the Michaeles-Menten equation at different concentrations of the Jacks Special Media mix (Table 2). *Ulva* on the spray system platforms (SHD, MHLD, BPVD) exhibited relatively higher uptake than the submerged controls (SUB). This is true for nitrate, ammonia, dissolved inorganic nitrogen and phosphate (Figure 5, 6, 7, 8). Ulva on platforms MHLD and SHD have higher preference for

nitrate uptake, while BPVD have more preference for ammonia and phosphate. As shown in the kinetic parameters, Ulva's maximum uptake rate and half-saturation value of nitrate on MHLD and SHD platforms is closely similar (Table 3). The use of different *Ulva* cultivation platforms effect uptake rates of these nutrients.

The difference in the mean dry biomass/day among the different levels of JSM is greater than would be expected by chance after allowing for effects of differences in the platform of cultivation and the different JSM levels: there is an observed statistically significant difference (P = <0.000) (Table 4). To isolate which group(s) differ from the others, a univariate multiple pairwise comparison procedure employed showed highest difference of dry biomass/day at 2X JSM with significant variation with 1X followed by 8X JSM (Table 5). Note that these mean difference comparison is significant at 0.05 levels. The JSM concentration and its interaction with the design as the factor showed significant effect on the mean dry biomass production per day of *Ulva lactuca* suggesting that specific nutrient levels is a controlling factor that led to the increase or decrease of the seaweed's growth rate.

Pigments analysis (Chl a, Chl b and carotene) were highest in the SHD platform, even better than SUB. This means that the pigments are not necessarily the controlling factor the directly affect the biomass build-up in *U. lactuca*.

The monitored pH, temperate, and light exhibits slight variations (Table 7). These co-variates will be considered in the correlation analysis of all treatments and variables with the protein production once we have the finalized protein production data.

4. Conclusion:

The Multi-level Horizontal design (MLHD) provided the highest growth rate of *Ulva lactuca* in a recirculating system using 2X Jacks Special Media (JSM). However, we concluded that the best growth rate can be obtained using glass type of material rather than using PE plastic containers. Based on these experimental results we project that improved growth rates and protein content should be obtainable under outdoor, higher solar light conditions as would be found in a greenhouse environment. We will further explore other input parameters (e.g., CO_2) to maximize growth and protein production of *Ulva lactuca* in spray recirculating system.

Development of new markets for cultured macroalgae will promote new environmentally friendly seaweed culture businesses (i.e., development of seaweed based shrimp aqua-feeds will increase the sustainability of the intensive shrimp culture industry and in for advancing inland aquaculture of shrimp.

5. Acknowledgements

This research was supported by Research Grant Award No. US-4599-13 R from BARD, The United States - Israel Binational Agricultural Research and Development Fund. We acknowledge the contributions of BARD partners Profs. Charlie Yarish & Jang Kim (University of Connecticut, Stamford), and to our student laboratory volunteer, Ms. Alyssa Velloze for her help tending the experimental systems and for data acquisition.

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Yong, Y.S., Yong, W.T. L, Anton ,A., 2013. Analysis of formulae for determination of seaweed growth rate. Journal of Applied Phycology 25:1831-1834.

FIGURES:

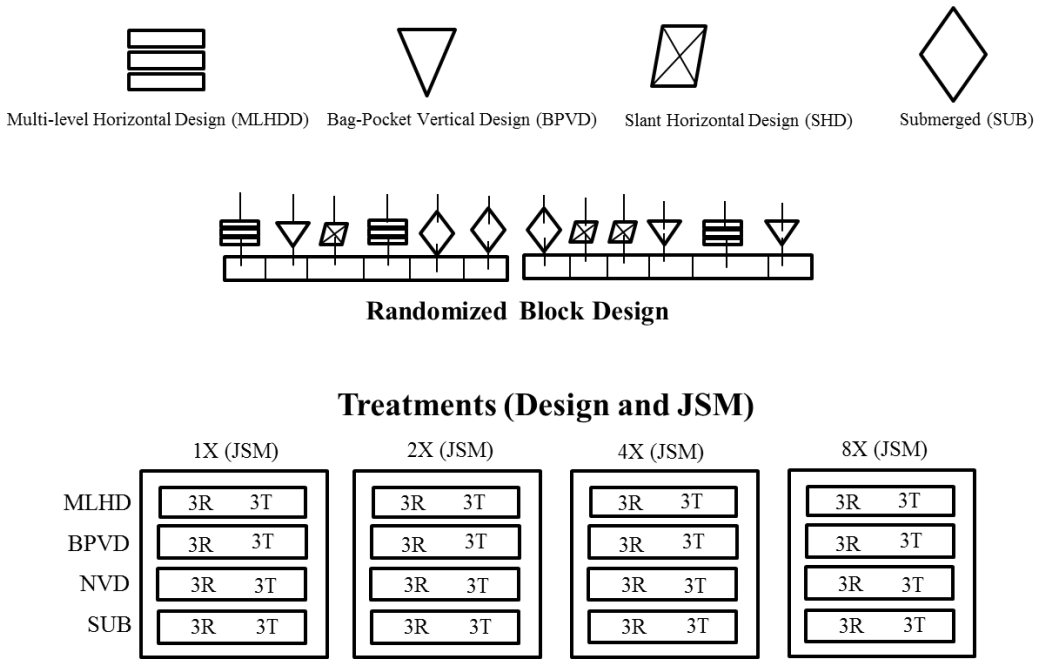

Figure 1. An randomized experimental design was employed to test the design and JSM concentration with growth performance of *Ulva* at alpha = 0.05. The study uses a two-factorial experiment to determine main effects and the interaction of these factors.

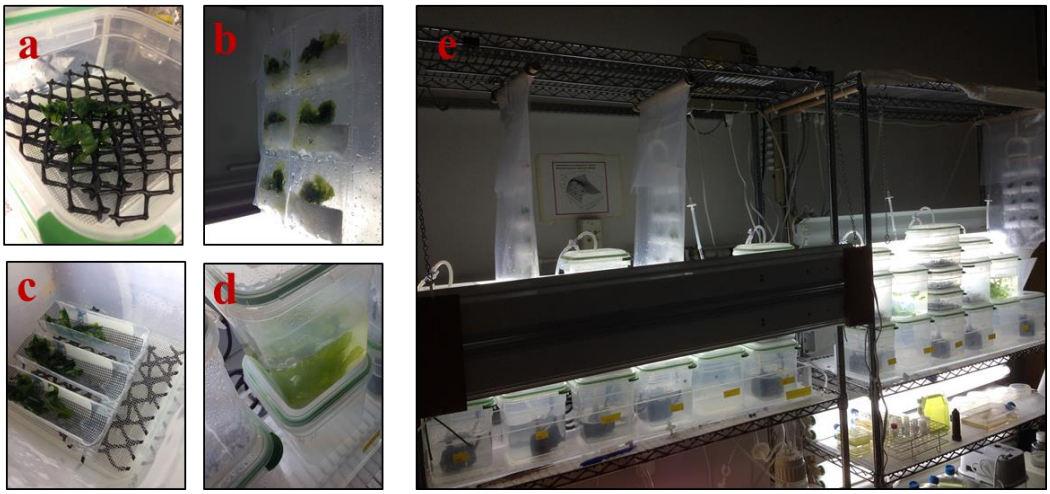

Figure 2. . Seaweed cultivation platform of *Ulva lactuca* grown at different JSM (mean ± stdev, n=3) concentrations on three different cultivation spray system for about 15 days. a) Multi-Level Horizontal Design (MHLD); b) Bag-Pocket Vertical Design (BPVD), c) Slant-Horizontal Design (SHD), d) Submerged (SUB).

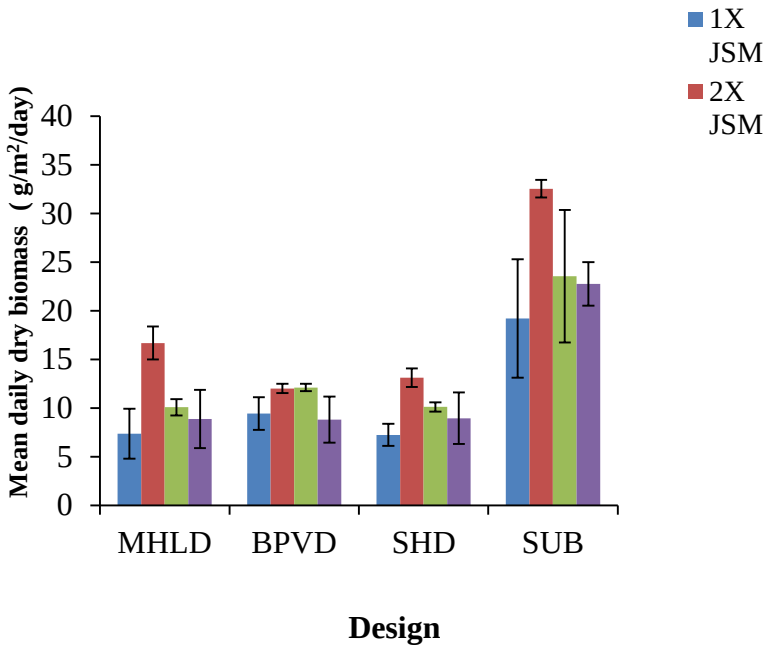

Figure 3. Mean biomass per day of *Ulva lactuca* grown at different JSM (mean ± stdev, n=3) concentrations on three different cultivation spray system cultivation for 15 days.

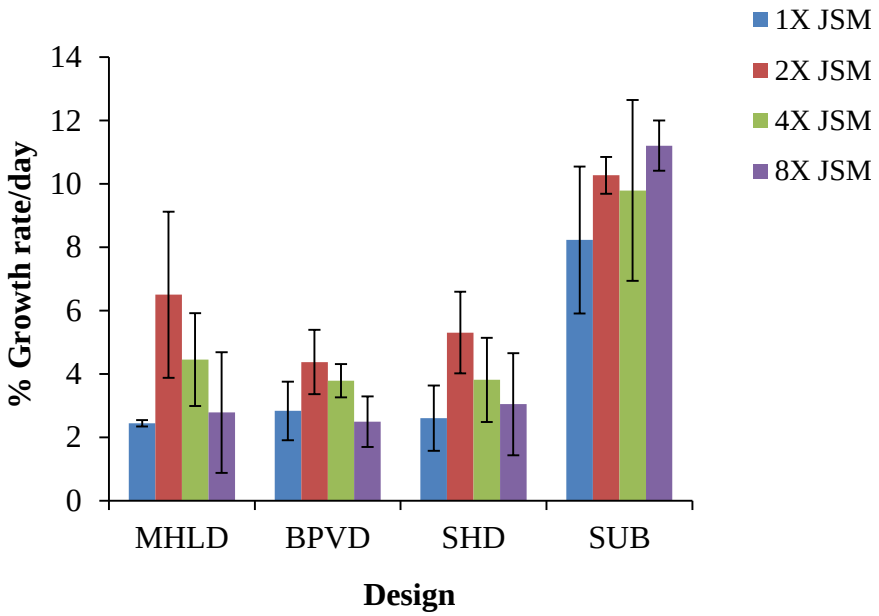

Figure 4 . %Growth rate per day of *Ulva lactuca* grown at different JSM (mean ± stdev, n=3) concentrations on three different cultivation spray system cultivation for 15 days.

Table 1. Water and AFDW content (ash-free dry weight) of *U. lactuca* (mean ± stdev, n=3) grown on different cultivation design-units (MLHD, BPVD, SHD and SUB) at different JSM concentrations.

Design	**Water content**	**AFDW**
	(%)	*g/m^2/day*
1X		
MHLD	74.53±4.95	5.72±0.14
PBVD	76.26±2.83	7.17±0.08
SHD	77.87±1.35	5.92±0.05
SUB	77.75±0.87	14.05±0.36
2X		
MHLD	77.37±1.19	13.18±0.02
PBVD	76.14±0.86	9.24±0.01
SHD	78.92±0.94	10.59±0.04
SUB	73.39±0.96	26.43±0.02
4X		
MHLD	79.67±1.37	7.93±0.02
PBVD	75.60±0.64	9.34±0.02

SHD	80.54±0.84	7.89±0.00
SUB	75.77±1.06	17.86±0.09
8X		
MHLD	81.17±2.65	6.98±.10
PBVD	75.34±5.32	7.22±0.10
SHD	78.62±3.18	7.85±0.08
SUB	80.06±0.83	17.16±0.04

Table 2. Nutrient concentrations of JSM on three different cultivation design-units (MLHD, BPVD, SHD and SUB).

Design	**NO3**	**NH4**	**DIN**	**PO4**
	µM	*µM*	*µM*	*µM*
Initial				
1X	252	166.8	419.2	30.8
2X	502	329.2	831.6	60.4
4X	1008	667.2	1676.8	123.2
8X	2440	1652.8	4099.2	328
SHD				
1X	0.27±0.04	0.26±0.01	0.55±0.06	2.10±0.57
2X	0.37±0.07	0.52±0.16	0.90±0.22	2.68±0.23
4X	87.77±15.49	2.31±0.80	90.76±14.25	11.84±1.17
8X	271.80±88.95	58.09±13.85	330.38±76.59	32.49±8.62
MHLD				
1X	1.03±1.121	1.42±1.17	5.40±6.11	15.39±12.20
2X	21.29±29.72	0.57±0.51	21.95±30.36	4.86±4.34
4X	67.27±5.48	1.44±0.58	47.01±37.91	5.49±4.84
8X	302.52±98.42	88.87±78.59	392.67±177.99	37.34±16.07
PBVD				
1X	1.44±0.51	1.54±0.42	2.98±0.94	19.44±7.27
2X	57.79±61.813	3.38±0.41	61.49±61.75	29.80±2.60
4X	615.10±206.30	7.35±0.62	625.66±206.94	66.84±22.39
8X	1861.59±347.60	214.28±57.39	2081.68±292.61	50.67±1.35
SUB				

1X	0.57±0.43	0.47±0.33	1.06±0.77	1.10±1.58
2X	1.69±0.56	2.06±0.64	3.87±1.23	5.70±1.89
4X	2.48±0.35	4.32±1.09	55.73±85.84	12.94±17.49
8X	7.42±1.61	497.73±40.34	508.19±39.12	2.78±0.20

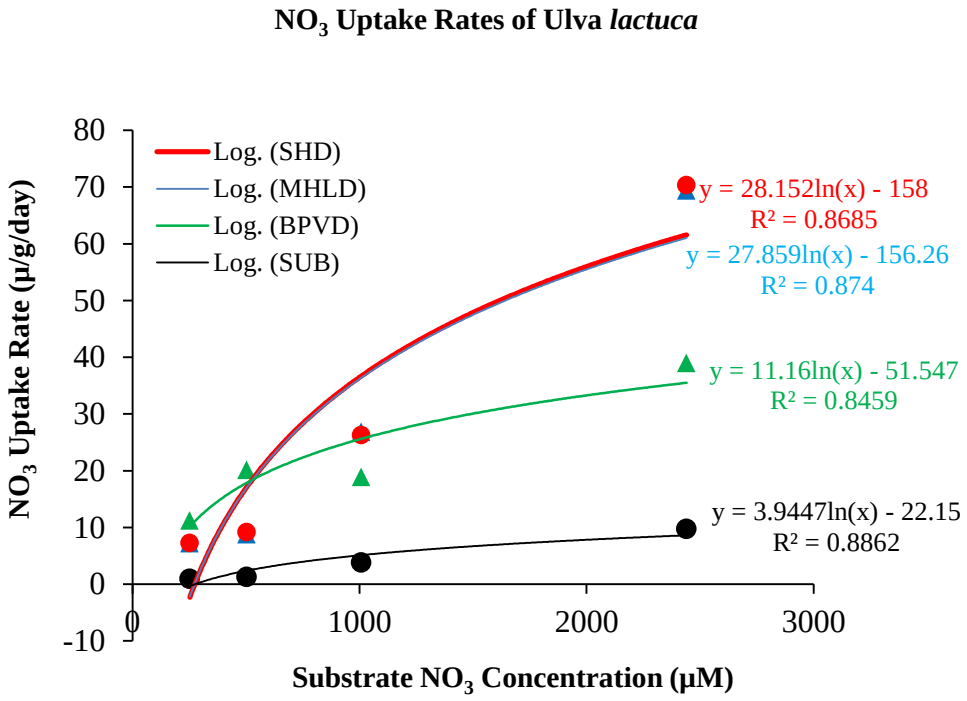

Figure 5. Parameters of the Michaelis–Menten function estimated by non-linear fitting of data from the four NO_3 uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

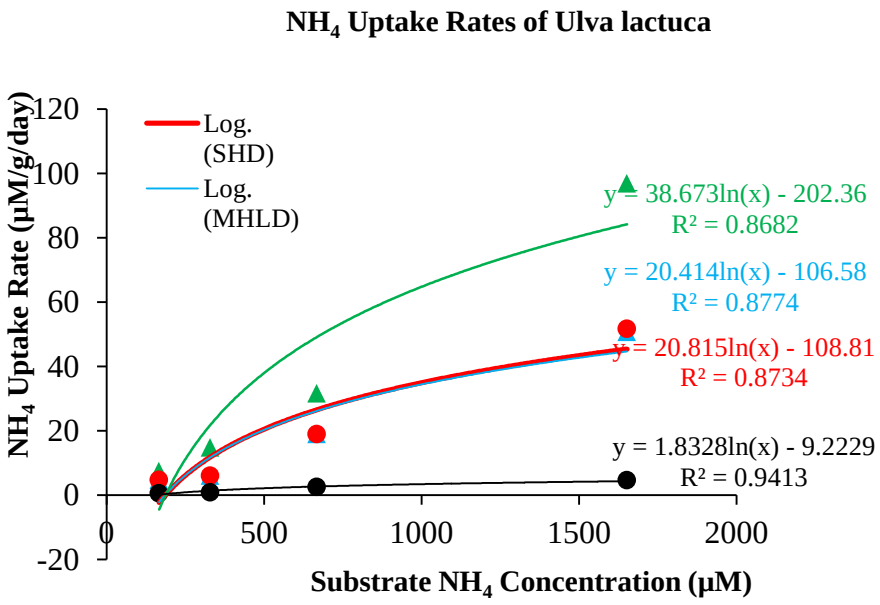

Figure 6. Parameters of the Michaelis–Menten function estimated by non-linear fitting of data from the four NH_4 uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

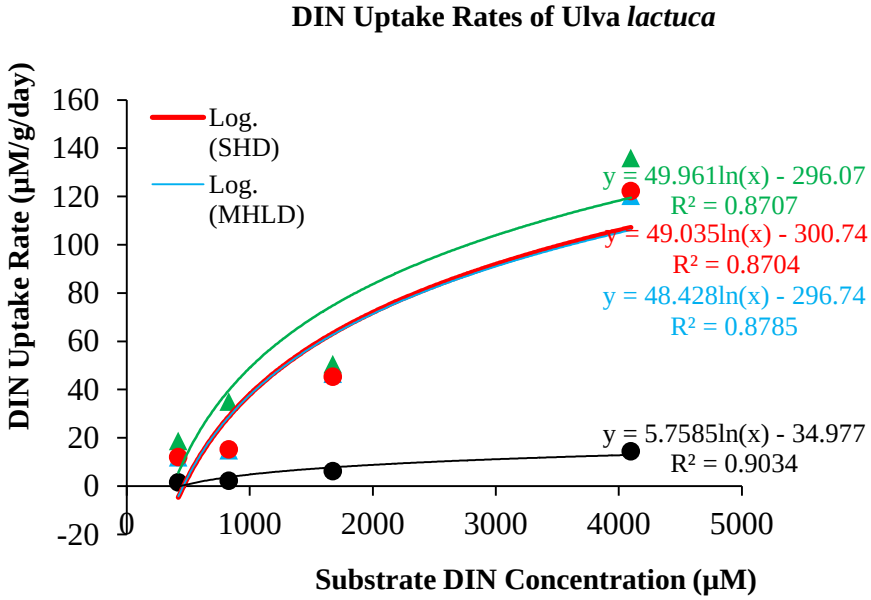

Figure 7. Parameters of the Michaelis–Menten function estimated by non-linear fitting of data from the four DIN uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

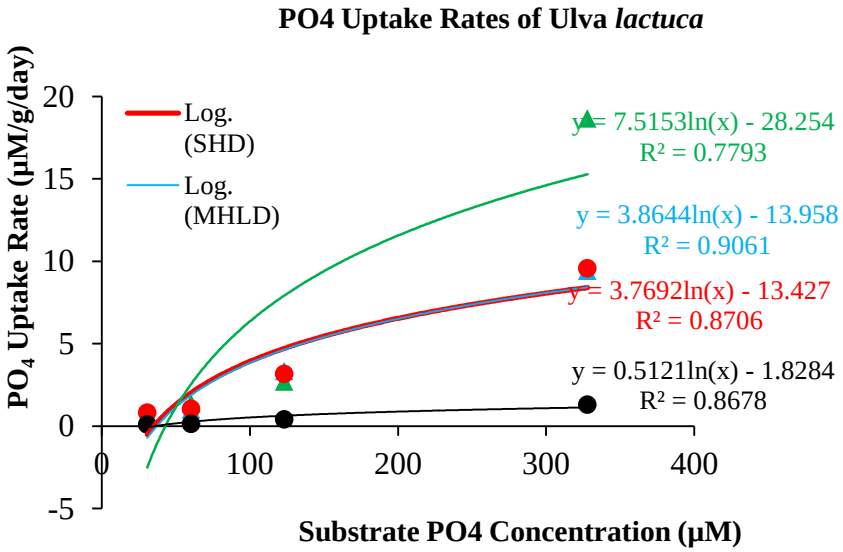

Figure 8. Parameters of the Michaelis–Menten function estimated by non-linear fitting of data from the four PO4 uptake experiments conducted with *U. lactuca* previously maintained in the laboratory with different concentration of JSM media.

Table 3. Kinetic parameters V_{max} (µmol N (g DW)$^{-1}$ h^{-1}), K_m (µM N) and affinity for uptake at low concentrations (V_{max}/K_m) for nitrate, ammonia, dissolved inorganic nitrogen and phosphate uptake in *U. lactuca.*

Design	NO_3			NH_4			DIN			PO_4		
	Vmax	*Km*	$\frac{VMax}{Km}$	*Vmax*	*Km*	$\frac{VMax}{Km}$	*Vmax*	*Km*	$\frac{VMax}{Km}$	*Vmax*	*Km*	$\frac{Vmax}{Km}$
SHD	61.57	817.35	0.075	45.43	554.94	0.082	107.16	1374.52	0.078	17.93	380.09	0.047
BPVD	35.51	497.27	0.071	84.22	553.12	0.081	119.53	1239.28	0.096	34.26	419.49	0.082
MHLD	61.03	815.95	0.075	44.69	556.36	0.151	106.11	1370.59	0.077	18.19	389.65	0.047
SUB	7.16	776.19	0.009	4.36	503.30	0.009	12.93	1334.43	0.010	2.43	381.64	0.006

Table 4. Univariate general linear models comparing interaction of effects of cultivation design and JSM concentration. The F tests the effect of 1. This test is based on the linearly independent pairwise comparisons among the estimated marginal means. Computed using alpha =0.05.

	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Contrast	392526.83	3	130842.28	24473.14	.000	.610
Error	251444.76	47031	5.35			

Table 5. Pairwise comparison using univariate general linear models comparing interaction of effects of design and JSM concentration.

Dependent Variable: Dry Biomass ($g/m^2/day$)

(I) 1	(J) 1	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
1	2	-7.964*	.031	.000	-8.025	-7.904
	4	-3.783*	.031	.000	-3.843	-3.722
	8	-2.158*	.031	.000	-2.218	-2.098
2	1	7.964*	.031	.000	7.904	8.025
	4	4.182*	.030	.000	4.123	4.240
	8	5.806*	.030	.000	5.748	5.865
4	1	3.783*	.031	.000	3.722	3.843
	2	-4.182*	.030	.000	-4.240	-4.123
	8	1.625*	.030	.000	1.566	1.683
8	1	2.158*	.031	.000	2.098	2.218
	2	-5.806*	.030	.000	-5.865	-5.748
	4	-1.625*	.030	.000	-1.683	-1.566

Based on estimated marginal means.* The mean difference is significant at the .05 level.
[b]Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 6. Chl a, chl b, total Chl and total carotene (mean ± stdev, n=3) grown in different cultivation design-units (MLHD, BPVD, SHD and SUB).

Design	**Chl a**	**Chl b**	**Total Chl**	**Total Carotene**
	mg/g	*mg/g*	*mg/g*	*ug/g*
1X				
MHLD	0.40±0.29	0.39±0.39	0.79±0.58	0.76±0.38
PBVD	0.37±0.34	0.35±0.33	0.72±0.67	0.76±0.30
SHD	0.67±0.38	0.65±0.31	1.31±0.69	0.78±0.25
SUB	0.13±0.12	0.14±0.14	0.27±0.26	0.40±0.23
2X				
MHLD	0.24±0.19	0.23±0.18	0.48±0.37	2.59±1.52
PBVD	0.13±0.05	0.12±0.04	0.25±0.09	1.56±0.41
SHD	0.29±0.11	0.27±0.10	0.56±0.21	1.96±0.39
SUB	0.14±0.07	0.13±0.06	0.27±0.13	3.14±1.81
4X				
MHLD	0.77±0.24	0.76±0.27	1.53±0.51	1.64±0.48
PBVD	0.93±0.02	0.86±0.03	1.79±0.06	1.39±0.39
SHD	1.01±0.38	0.90±0.36	1.91±0.74	1.20±0.12
SUB	0.49±0.15	0.49±0.15	0.98±0.30	0.52±0.06
8X				
MHLD	0.07±0.09	0.06±0.08	0.13±0.17	0.34±0.51
PBVD	0.64±0.19	0.60±0.17	1.24±0.36	0.66±0.49
SHD	0.90±0.43	0.83±0.40	1.73±0.83	0.80±0.56
SUB	0.74±0.08	0.66±0.09	1.40±0.17	1.58±0.66

Table 7. Culture conditions of *Ulva lactuca* grown in three different cultivation designs.

		pH	**Temp (^{o}C)**	**Light (µE/m^2/s)**
1X	*MHLD*	8.42±0.01	26.57±0.21	202.00±48.48
	BPVD	8.47±0.03	25.90±0.40	157.73±16.83
	SHD	8.36±0.09	27.10±0.61	218.61±60.05
	SUB	9.05±0.72	26.23±0.68	251.81±31.15

2X	*MHLD*	-	27.27±0.64	118.07±3.20
	BPVD	-	26.30±0.44	136.51±27.86
	SHD	-	27.27±0.51	146.66±33.55
	SUB	-	26.77±0.40	140.20±36.85
4X	*MHLD*	8.59±0.09	28.80±0.26	196.57±17.29
	BPVD	8.57±0.13	26.63±0.15	164.27±26.89
	SHD	8.46±0.03	28.67±0.32	157.81±54.46
	SUB	9.98±0.13	25.90±0.53	219.64±6.39
8X	*MHLD*	8.01±0.17	29.50±0.53	175.25±20.95
	BPVD	7.81±0.12	27.47±0.57	171.56±5.53
	SHD	7.89±0.16	28.57±0.75	199.24±28.76
	SUB	9.69±0.17	25.93±0.40	215.84±59.86

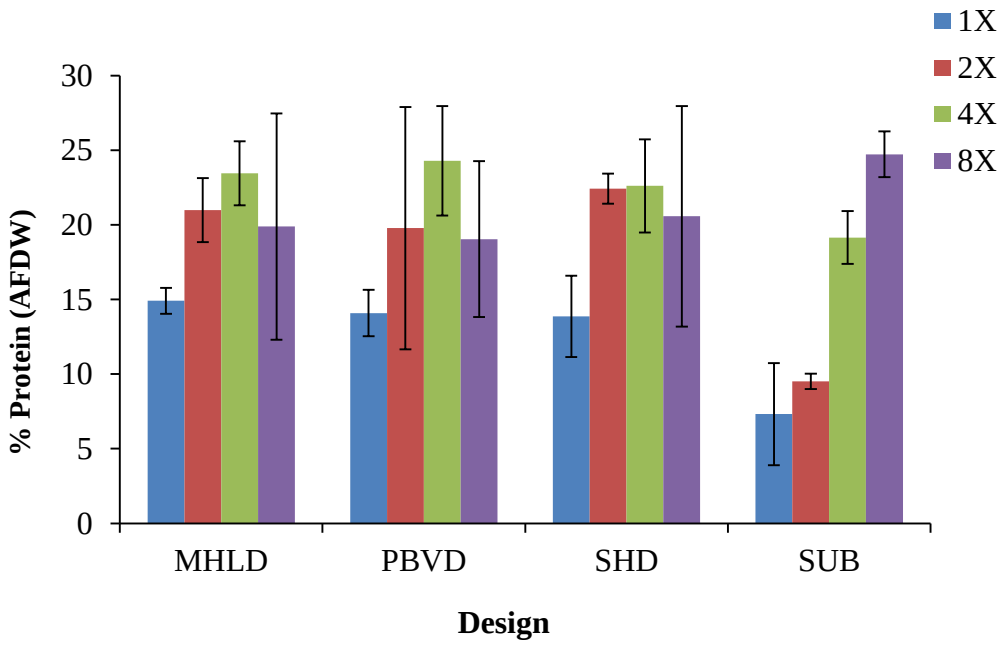

Figure 9. Percent (%) Protein content of *U. lactuca* grown at different concentration of JSM media.

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3) Partner Organization: Israel Oceanographic and Limnological Research, National Center for Mariculture (IOLR-NCM)

Task / Objective: Objective 3. Cultivate several species in kg quantities (dw) for use in experimental diets for marine shrimp.

Cover Page

BARD Project Number: US 459913 R

Date of Submission of the report: June 30, 2017

Project Title: The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aqua-feeds

Investigators	Institutions
Principal Investigator (PI): Amir Neori	**IOLR-NCM**

Co-Principal Investigator (Co-PI): Lior Guttman **IOLR-NCM**

Collaborating Investigators:

Keywords *Ulva*, *Hypnea*

Abbreviations commonly used in the report, in alphabetical order**:**

Budget: IS: $ 136,800 US: $ 277,900 Total: $ 414,700

Signature	Signature
Principal Investigator	Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1, with Samocha			1
Submitted, in review, in preparation			2	2
Invited review papers				
Book chapters			1	1
Books				
Master theses			1, Y. Bronfman	1
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings				
Longer Visits (Sabbaticals)				

Description Cooperation:

Algae from Connecticut to Israel: Several strains of different algae were brought to Israel by C Yarish UCONN in 2015. Half of the samples were maintained in Haifa, in Dr. Alvaro Israel's lab, in incubators. The other half of the samples was brought to Eilat. These were maintained in filtered running seawater under artificial light (12 h on and 12 h off). Unfortunately all the strains did not survive, we do not know the reason.

Several kg of dried Ulva sp. algae from production systems were shipped to Texas, for experimental diets for shrimp.
Several kg of dried Ulva sp. algae from production systems were shipped to Alabama, for experimental diets for shrimp.

Patent Summary (numbers) **NONE**

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract: An alternative method for seaweed land-based culture - spray/drip culture, was studied. In theory, this method reduces construction cost energy demand, compared to ponds. The farms can be located anywhere, with minimal site preparations. The seaweed grow on light exposed surfaces, in a minimal layer of culture water, which can be nutrient-rich fishpond effluent or fertilizers.

The following objectives were defined in the proposal for the research in Israel:

Objective 2. Establish defined enriched seawater growth media for macroalgae that will induce increased protein content. Task 2.1. From the above starter material, UCONN, UCSD SIO and **NCM** will test the effects on protein content of several ammonia and nitrate concentrations as well as combined day-length and temperature.

Achievements in Israel: We have used several media, based on fishpond effluents and their enrichment, studied the effect of nutrition on the chemistry of several "Israeli" algae. The strains from C:\uconn*UCONN didn't survive long enough to do that.*

Objective 3. Cultivate several species in kg quantities (dw) for use in experimental diets (**NCM**). In Israel, the couple of best performing strains from UCONN/UCSD-SIO testing (plus a strain selected locally) will be grown in larger spray-culture units in a greenhouse (to be constructed at NCM) and tested against each other for overall growth and biomass performance, and protein-lipid spectrum and content. Seawater and nutrients for the spray-culture growth trials will be supplied from near-by fish ponds and piped/pumped to the spray-culture greenhouses. Controlled algal biomass production of the greenhouse-cultured macroalgae will be conducted using seawater, enriched by either fertilizers or fish-pond/shrimp-pond effluent (at NCM).

Achievements in Israel: We have developed and used several models of spray/drip irrigation culture with several species of seaweeds, as proposed, within the thesis of Mr.

Bronfman, and in other experiments. We produced and sent to the USA (TAMU and Auburn) several kg of dries seaweeds, which varied in their protein content.

Specific Results Expected from this Work (as listed in the accepted proposal for funding):

1. Availability of seaweed strains that can be spray-cultured in large quantities and produce high levels of cellular proteins.

Achievements in Israel: We have studied and identified a couple of strains, namely Ulva compressa and Hypnea musciformis, as two seaweeds that performed particularly well under spray/drip conditions.

3. The cost of the cultured seaweed biomass has the potential to be reduced significantly, thanks to the novel spray culture approach, in comparison to other forms of inland seaweed culture.

Achievements in Israel: We have evaluated the economics of the system, and defined conditions under which it has advantages over the standard suspension (tumble) culture.

4. The use of energy in the culture will be smaller than with the tank and pond culture.

Achievements in Israel: We have calculated that under Israeli conditions, spray/drip irrigation reduces energy consumption of the culture itself by over 20%.

5. The culture system will take up significant quantities of waste nutrients, including nitrogen and CO_2.

Achievements in Israel: Nutrient uptake rates were measured.

6. The design of the culture system will be scalable to commercial scale.

Achievements in Israel: we have developed and examined a tile-based system that is easy to install and operate on any scale.

Conclusions and major achievements: Several species of seaweed were very suitable for this approach and provided good yields of a good quality biomass. The tile culture system promises to be the basis of large -scale low-cost farms, anywhere, but it is not ready for that yet.

Yossi Bronfman, studying for an MSc degree in the Hebrew University of Jerusalem) (Fig. 1).

The units were fed by surface seawater. An outdoor 9-unit $1m^2$ (each unit) seaweed production system, aimed to grow kilograms of macroalgae for the shrimp feed diets, has been designed, built and operated (photo appendix Figs 2, 3). The units are spray-fed fishpond (with the fish grey mullet) effluents.

Summary of Year 1 achievements in Israel

Two efforts have progressed toward the objectives:

1. An experimental outdoor spray irrigation system has been designed, built and used (by Mr. for about a week or 10 d, each tank is harvested, rinsed and shade-dried for several days (Fig. 3). Then the dry biomass from the 9 tanks is crushed, and after sampling for analyses, put in a bag for shipment.

2. An existing greenhouse was renovated, and then used in the tile spray/drip -culture experiments.

3. A shipment of dried samples for analysis and for consideration in experimental shrimp diets has been shipped from NCM to TAMU.

Year 2 achievements in Israel

This year's efforts have made further progress toward the sated objectives:

1. The outdoor seaweed production spray irrigation system, which had been completed and reported in 2014, produced poor algae, mixed with silt and with around 10% protein in dw, due the availability at NCM of only a silty effluent with low nutrient content. With the advice

of Prof. Samocha, a nutrient enrichment system equipped with water filtration - was designed and built (Fig. 4).

2. The modified seaweed production system produced a better quality biomass. A shipment of about 7 kg of *Ulva* sp. meal, with a protein content around 25% in dw, was sent in summer to the new site of the shrimp feeding trials, in Auburn AL. A second shipment of a similar size was shipped in October, with over 30% protein in dw. Total *Ulva* meal prepared this year was 15 kg.

   A novel experimental tile-based spray/drip irrigation system was designed and built in the greenhouse (following its renovation in summer 2014, Fig. 5, 6).

   The tile system was smaller than the outdoor production system. However, its location in the greenhouse and its connection to the supply of nutrient-enriched fishpond effluents allowed algae production also in winter.

3. Two algae, an *Ulva* and two *Gracilarias* - brought by Prof. Yarish from UCONN (see UCONN report) - were placed for growth in a culture room. While at first both algae grew, in early summer both died, apparently between other factors due to the heat, as the culture room available to us was not air-conditioned.

Year 3 achievements in Israel

1. Mr. Yossi Bronfman, who, with the funding by this project, has studied the spray/drip method for his MSc degree in the Hebrew University of Jerusalem, has completed his studies, and his degree was confirmed **with a final grade of 91 (A[8]),** on cultivation of several macroalgae species in spray culture. Below is a brief report on these experiments:

My research examined an alternative method for seaweed land-based culture - spray/drip culture. It has several theoretical advantages compared with suspended (tumble) seaweed culture, including lower cost for construction and operation energy, and a site flexibility. Spray/drip culture farms, even on a large scale, require little leveling of the ground. Published data from this laboratory and others have suggested, however, that the areal yield in spray/drip seaweed culture was below suspension ponds by several tens of percent.

[8] See an appendix

The performance of the seaweed production spray/drip culture, fed nutrient-rich simulation solution of effluents from intensive fishponds, as the plant component of IMTA (integrated multi-trophic aquaculture), was evaluated. Several experimental systems were built and used to select suitable algae, and define the operational parameters, toward commercial feasibility study. The impact of irradiance and water layer thickness on SGR[9] and nutrient uptake of *Ulva fasciata* grown in net-mattresses on trays (Fig. 7), was compared with ponds, with respect to tray slope and irradiance. Another series of experiments examined SGR, nutrient uptake, protein content and general quality of the algae yield (based on visual and biochemical parameters, Fig. 8). *U. fasciata* grew best and with the best quality with 6^0 slope (53 g m^{-2} d^{-1} (yields are in fresh wt), compared with 120 g m^{-2} d^{-1} in ponds. Protein content was highest in 80^0 trays, but growth with this slope was poor.

In Another series of experiments SGR, nutrient uptake, protein content and quality of *U. fasciata* were studied in 6^0 with respect to nutrient (N, P) enrichment level (Fig. 8). Yield correlated with the level of nutrient enrichment and reached 84 g m^{-2} d^{-1}, compaed with the yield in control ponds of 100 g m^{-2} d^{-1}, with the same protein contents for both. Similar experiments were conducted with *Hypnea musciformis* (Fig. 9) and *U. compressa*. Yield of *H. musciformis* reached 286 and 319 g m^{-2} d^{-1}, in spray culture and ponds, respectively, and yield of *U. compressa* reached 172 and 97.5 g m^{-2} d^{-1} in spray and pond, respectively.

2. Several experiments examined the spray/drip irrigation culture of *Ulva* sp. On cement and ceramics tiles in the greenhouse (Fig. 5, 6), nutrient enriched and filtered fishpond effluents:

The experimental tile system was technically easy in its operation. However, the results varied. Yield ranged from zero to about 50 g fw (7 g dw) m^{-2} d^{-1}. Protein content of the yield ranged from 30 to 44% in dw. Lipid content ranged from 2.5 to 4.5 % in dw, a high value that requires a reexamination. The nutrient data were not good enough for calculations of nutrient uptake rates.

CONCLUSION: the spray culture method is not suitable for all algae. Three species grew successfully with reasonable yield and crop quality, and took up nutrients efficiently. *U.*

[9] SGR= specific growth rate

compressa was the only species of those studied that actually preferred spray culture. Both *H. musciformis* and *U. fasciata* grew well in the experimental spray culture systems, although growth rate was slower than in ponds. It was observed, and requires more study, that high irradiance and faster equilibration with air temperature can impede some algae, when irradiance is high and temperature is much different form the optimum for the species.

A preliminary business plan and economical calculations have suggested, that savings in construction and operation more than make up for higher labor costs that are involved with spray culture of seaweed, so that overall the cost of spray culture production per ha is lower by several hundred thousand NIS, compared with pond seaweed farming.

The research suggests that spray culture seaweed farming can be efficient, economic and reliable. Seaweed biomass produced in this technology is similar in quality to seaweed from conventional seaweed ponds. With more work, and under conditions that avoid extreme air temperatures, spray system could supply the markets seaweed with reasonable quality, quantity and low price.

References Cited:

Robertson-Andersson, D. V., Wilson, D. T., Bolton, J. J., Anderson, R. J., & Maneveldt, G. W. (2009). Rapid assessment of tissue nitrogen in cultivated *Gracilaria gracilis* (Rhodophyta) and *Ulva lactuca* (Chlorophyta). *African Journal of Aquatic Science*, *34*(2), 169-172.

Publications resulting from this research:

1. **Joint IS/US authorship, a published article**
2. Samocha, T. M., Fricker, J., Ali, A. M., Shpigel, M., and Neori, A. (2015). Growth and nutrient uptake of the macroalga *Gracilaria tikvahiae* cultured with the shrimp *Litopenaeus vannamei* in an Integrated Multi-Trophic Aquaculture (IMTA) system. *Aquaculture*, *446*, 263-271.

3. Israel: Submitted Articles

a. Milstein, A., Levy, A., Neori, A., Harpaz, S. & Guttman, L. (submitted to Aquaculture). Marine periphyton system for mariculture effluents treatment and food source for marine fingerlings: water quality in the periphyton compartment.

4. Israel: Thesis

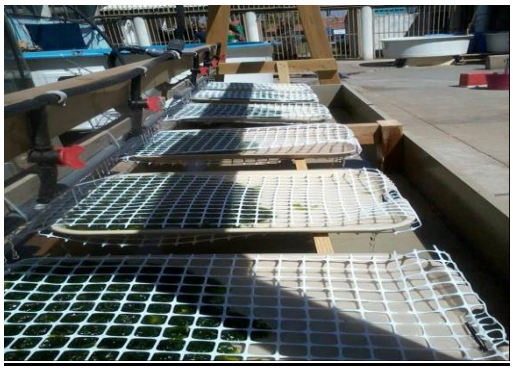
   a. Bronfman, Y. (2016). The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aqua-feeds. M.Sc. Thesis, Submitted to the Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem.

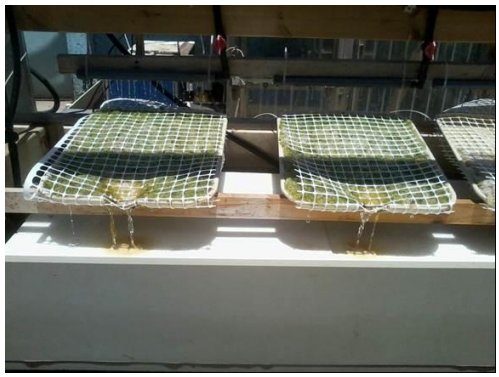
5. Israel: Book Chapter

   a. Neori, A., Shpigel, M. & Israel, A. (in press). The development of integrated multi -trophic aquaculture (IMTA) in Israel. Chapter 5 in: *Greening the Blue Revolution: the Turquoise Revolution of Integrated Multi-Trophic Aquaculture (IMTA),* T. Chopin, A. Neori, S. Robinson and M. Troell (Eds.), Springer Publishers, Dordrecht.

Photos - Appendix

Figure 1: An experimental spray-irrigation system. Each of the 6 trays has a culture area of 0.12 m^2. Note the water spray at the top of each tray, with drainage at the bottom. Three of the trays are perfectly horizontal, the other trays are slanted at a 6^o angle.





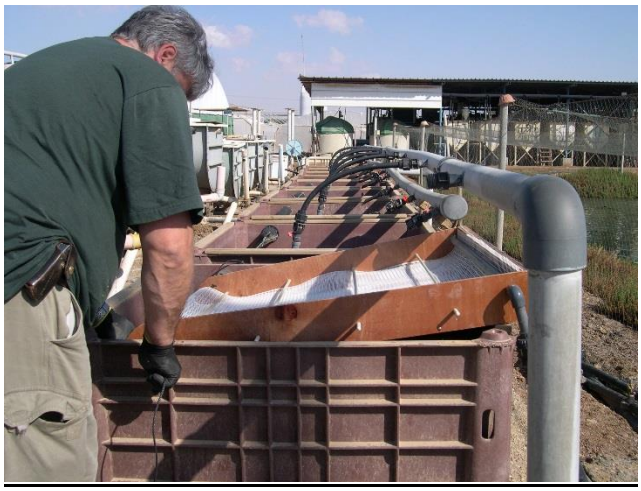


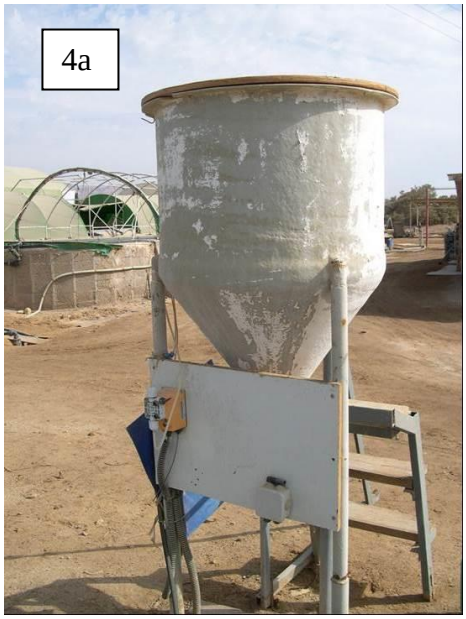
Figure 2: The large scale production spray-irrigation culture system, during construction. Each of nine units has an area of 1 m^2.

Figure 3. The production spray-irrigation culture system. 3a. The units with a ready-to-harvest crop of locally-grown *Ulva lactuca*. The water source was the sump pond in the background right. 3b. Air-drying rinsed *Ulva* in the greenhouse.

3a

3b

Figure 4: A water filtration nutrient- enrichment system for the water supply of the seaweed production units. 4a, a 400 L nutrient solution tank with a (yellow) dosing pump. 4b, an agricultural water filter (100 micron mesh).



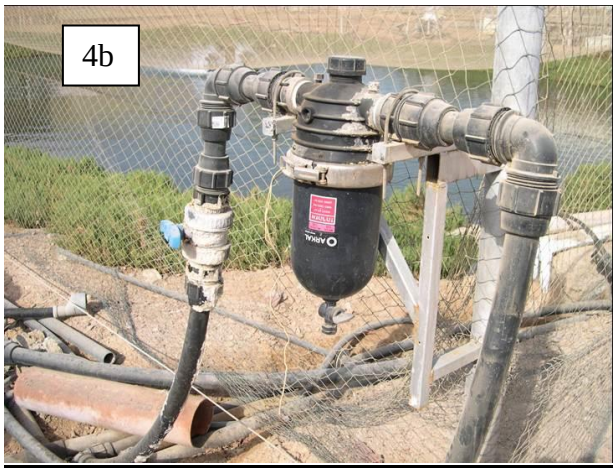
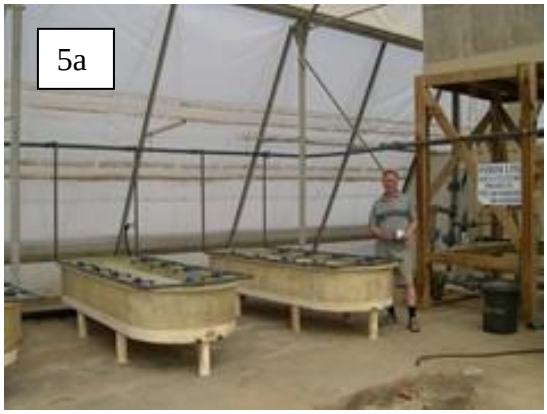


Figure 5: An experimental tile (cement) spray/drip -irrigation culture system in the renovated greenhouse. Three oval fiberglass ponds, each containing eight cement tiles, each tile with a culture surface area of 0.2 m^2, a total of 1.6 m^2 per tank.
5a, system construction in the renovated greenhouse.
5b, the entire system.
5c, upon stocking, May 2015.
5d, after two weeks of growth.



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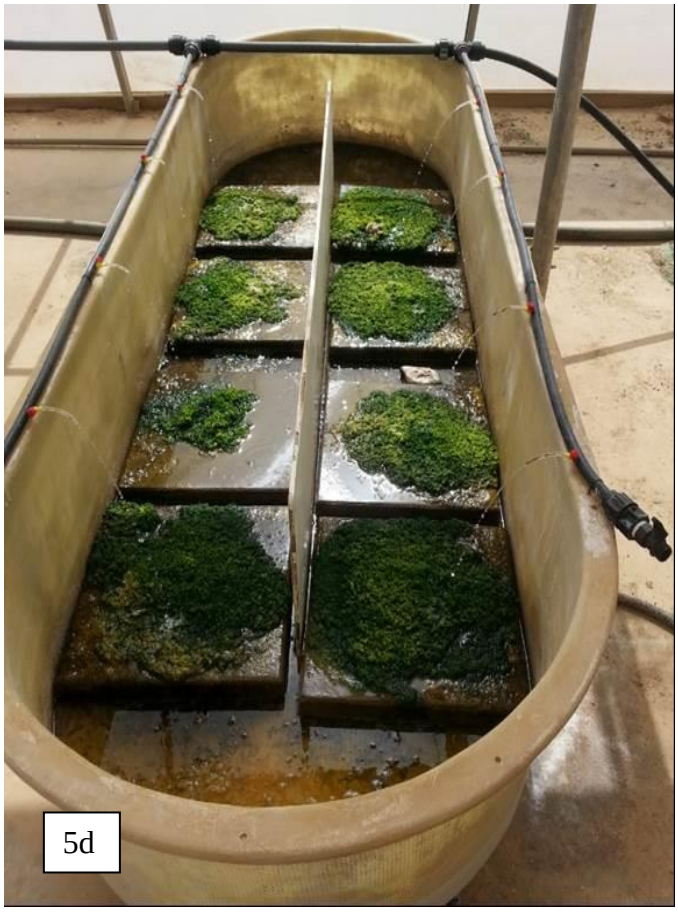


5d

Figure 6: An experimental tile (ceramics) spray/drip -irrigation culture system in the renovated greenhouse.

6a, stocking day.

6b, growth after 10 days.

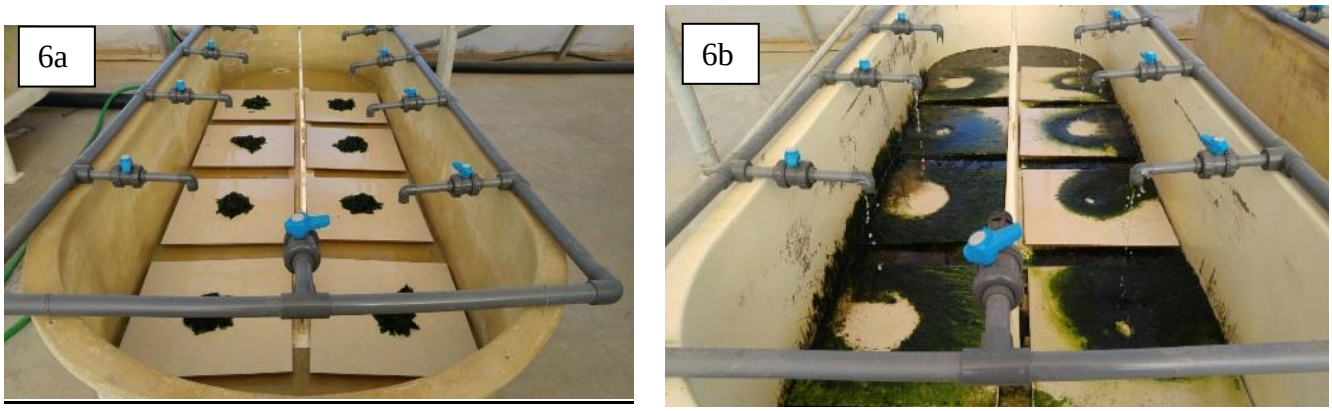


Figure 7: An experimental spray/drip culture system, with *Ulva fasciata*.
7a, seaweed mattresses; on left upper side of the picture, the small nutrients pipe and the regulator can be noticed. 7b, a control seaweed suspension culture tank.

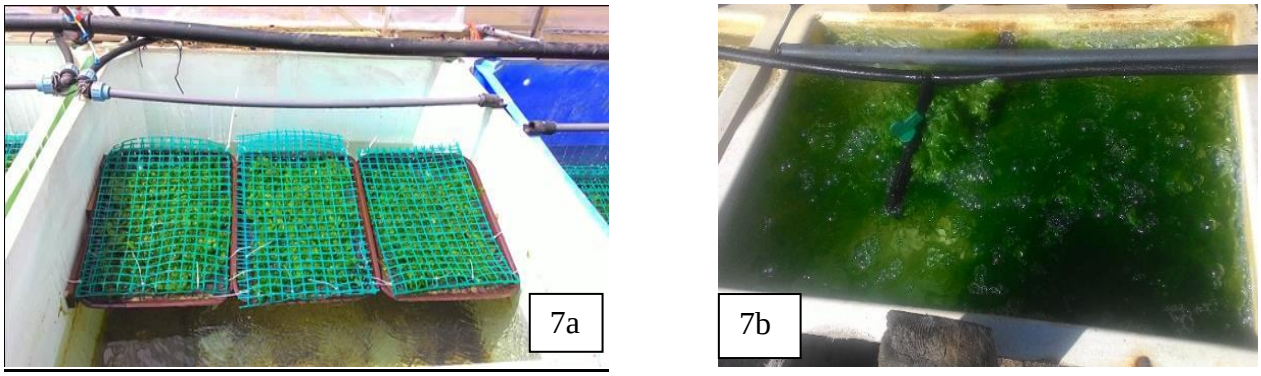


Figure 8: A color key for *Ulva* (following Robertson-Andersson et al., 2009)

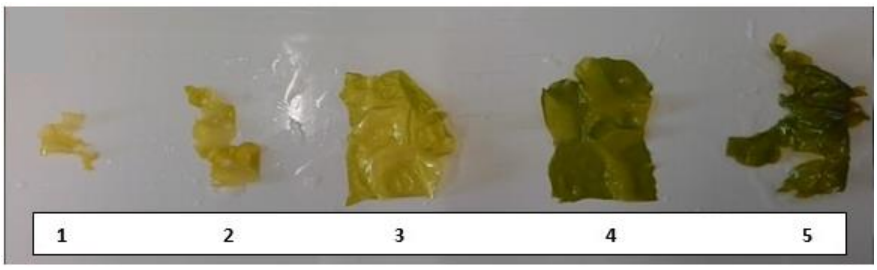
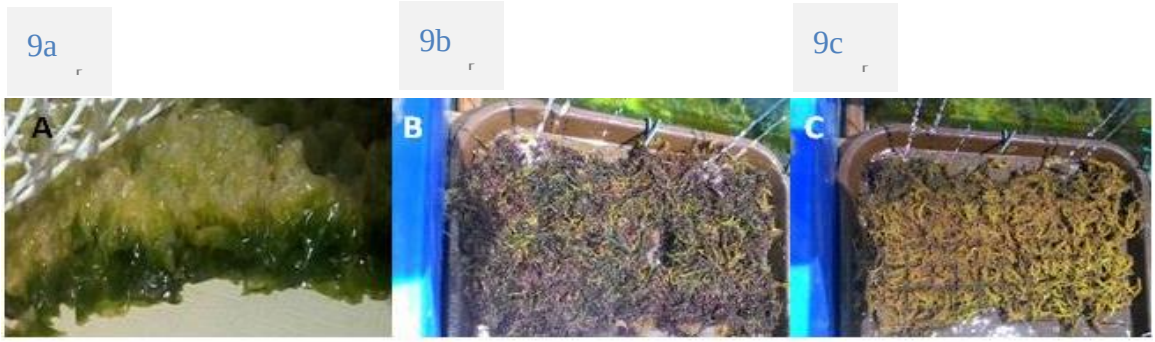


Figure 9: Layering in spray/drip -irrigated seaweeds.

9a, *Ulva fasciata*.

9b, ***Hypnea musciformis***- deeper layer, less exposed to light and air.

9c, ***H. musciformis*** upper layer, fully exposed and bleached.



The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aquafeeds. -- BARD Project Number: US-4599-13R

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Aquaculture

Manuscript Draft

Manuscript Number:

Title: Marine periphyton biofilters in mariculture effluents: nutrient uptake and biomass development

Article Type: Research Paper

Keywords: Mariculture, Periphyton, Effluent treatment, Nitrogen removal

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Abstract: Cost of effluent treatment, water quality maintenance and feeds constitute most of aquaculture's operational costs, and influence its sustainability. The present study examined the effectiveness of periphyton for biofiltration of mariculture effluents. Marine periphyton was allowed to spontaneously develop on a plastic net substrate, in experimental bioreactors that were fed with effluents from fish mariculture ponds. Biomass development and nutrient uptake were followed in four seasons. Attention was given to the orientation and area of the net substrate, season and additional operational factors. The highest specific growth rate (SGR) of 27% day-1 was measured during the second week of periphyton growth in summer. Mean daily periphyton production rates in spring and autumn were 2.4 and 1.8 g (ash- free dry weight) m-2 day-1, respectively. The vertical orientation of the net substrate was overall advantageous over the horizontal orientation. Increasing the substrate area of vertically oriented nets in the biofilter increased the removal efficiency of total ammonia nitrogen (TAN) up to 80%, and allowed more biomass production in the biofilter. Multiple polynomial regression models suggest strong effect of biomass weight and effluent retention time on the removal efficiency of TAN and dissolved inorganic nitrogen (DIN). The removal rates TAN and DIN in these experiments were between 0.11 - 1.2 g N m-2 (substrate area) day-1 for periphyton at the age of 7 and 42 days, respectively. Marine periphyton seems to be a simple, low cost and sustainable component, which can perform biofiltration/rehabilitation of water quality and potentially serve as feed for fish and shrimp.

The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aquafeeds. -- BARD Project Number: US-4599-13R

Manuscript number	AQUA_2016_352
Title	Marine periphyton system for mariculture effluents treatment and food source fo marine fingerlings: water quality in the periphyton compartment
Article type	Research Paper

Abstract

The present study is a segment of a research project to study the biofiltration functioning of periphyton in maricultur effluents while concomitantly serving as food for marine fingerlings. The experimental system consisted of three fishponds, twelve periphyton biofiltration tanks, a mechanical bead filter and a nutrient fertilization system. The objectives of this study were to identify the main ecological processes and management procedures that affected water quality in the periphyton biofiltration compartment. The data collected were subjected to the multivariate statistical technique of factor analysis. Three factors accounted for most of the variability of the data: biological activ (periphytic organic matter accumulation, photosynthesis, and nutrient uptake), autotrophic biomass density and nitri and phosphate uptake / release balance. The results were utilized to construct a conceptual model that describes th ecological functioning of the periphyton biofilters. The delicate equilibrium that developed among the different processes in the periphyton compartment has implications for the management of the system to fulfill its double objective. As periphyton food source for marine fingerlings the target is to produce large amounts of periphyton of th appropriate quality. As a biofilter for mariculture effluents, the maintenance of an appropriate water flow regime is a key management element. An excessive water flow rate (a short water retention time) saturates the biofiltration capacity of a given periphyton system, overloads it with organic matter from the fish tanks that gradually clog the wa supply system, and washes out nitrifying particles promoting nitrite accumulation. On the other hand an insufficient water flow rate leads to a high efficiency of nutrient uptake by the periphyton, but reduces the overall nutrient uptak rate and also enables particle sedimentation, which promotes decomposition and nutrients release (instead of removal). The challenge is to find the appropriate balance among water flow rate, line cleaning frequency, substrate density and the biological processes developing in the periphyton compartment that produce adequate amounts of periphyton to feed marine fingerlings while optimizing nutrient removal.

Keywords	biofilter; mariculture effluents; periphyton ecology; water quality.
Taxonomy	Aquaculture, Biofiltration
Manuscript category	Production science

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The use of aquaculture effluents in spray culture for the production of high protein macroalgae for shrimp aquafeeds. -- BARD Project Number: US-4599-13R

Chapter 5. The Development of Integrated Multi-Trophic Aquaculture (IMTA) in Israel

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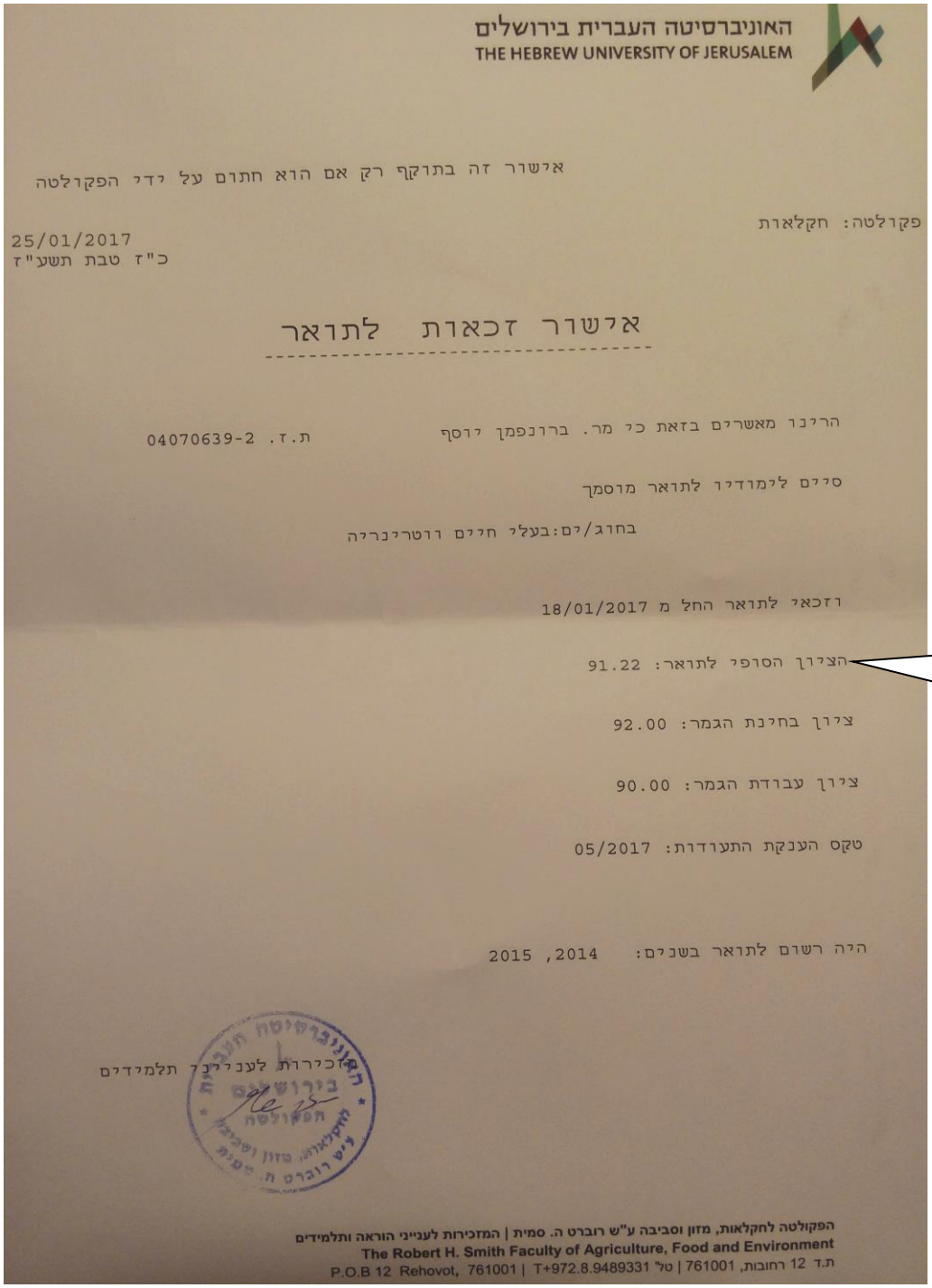
Abstract. Israeli aquaculture begun with greenwater culture of the common carp in the 1940s. Polyculture with tilapia, grey mullet and planktivorous carps followed. Scientific research on polyculture started in the 1950s. The Israeli research has since contributed to the global science and practice of greenwater and polyculture approaches and concepts. Today the industry is characterized by freshwater polyculture, utilizing intensive fishponds and reservoirs for semi-intensive freshwater aquaculture. In the Mediterranean coastal plain, fresh-, brackish- and marine- water polyculture is carried out in semi-intensive fishponds. Polyculture in Israel is an entrepreneurial activity that combines the ecological principles of the Chinese polyculture system with the local technologies and objectives (profit and dividends) of intensive industrial fish production systems. Biofloc approach (active suspension ponds, ASP), periphyton, and aquaponics were developed starting in the 1980s, responding to rising public and policymakers' concerns and regulations about land use, pollution, and use of chemicals and manures. R&D on marine integrated multi-trophic aquaculture (IMTA) systems began in the early 1970s at the National Center for Mariculture (NCM) in Eilat. It started with sea bream and mullet in green/brown-water earthen ponds, whose water recirculated through bivalve and macroalgae biofiltration / culture units. The concept was made modular starting in the early 1980s, and its functioning was studied in detail using nutrient budgets. The nutrient pathways were quantified, and several models were proposed and examined on small and pilot scales. Abalone, sea urchins, shrimp, brine shrimp and *Salicornia* were added to the Eilat IMTA models, starting in the 1990's. Upon entering the third millennium, Israeli research further examined the relationship between sustainability and economics in world aquaculture, and proposed a considerable role for IMTA in achieving global food security later this century.



האוניברסיטה העברית בירושלים
THE HEBREW UNIVERSITY OF JERUSALEM

אישור זה בתוקף רק אם הוא חתום על ידי הפקולטה

פקולטה: חקלאות

25/01/2017
כ"ז טבת תשע"ז

אישור זכאות לתואר

הרינו מאשרים בזאת כי מר. ברונפמן יוסף ת.ז. 04070639-2

סיים לימודיו לתואר מוסמך

בחוג/ים:בעלי חיים ווטרינריה

וזכאי לתואר החל מ 18/01/2017

הציון הסופי לתואר: 91.22

ציון בחינת הגמר: 92.00

ציון עבודת הגמר: 90.00

טקס הענקת התעודות: 05/2017

היה רשום לתואר בשנים: 2014, 2015

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Final overall grade

4) BARD Project Partner – Auburn University, Department of Fisheries and Allied Aquacultures (AUBURN)

Objective 4. Design and prepare experimental seaweed-based pelleted shrimp diets based on the composition of commercial shrimp diets and the composition of the produced macroalgal meals.

Task 4.1. To determine the ability of macroalgal meal to replace the alginate binder, test diets will be formulated with seaweed meals (incorporated at expected fishmeal replacement levels) and 2, 1 or 0% alginate binder. Feed water-pellet stability will be measured.

Task 4.2. To ensure diets are optimally formulated, protein and energy digestibility will be determined for the protein enriched seaweed meals prior to dietary formulation. These studies are scheduled to start only in the second year.

Objective 5. Conduct shrimp feeding trials with the experimental macroalgae diets in comparison with a commercial aquaculture shrimp aqua-feeds. These studies are scheduled to start late in the 2nd year, and continue on extension in the 3rd year under direction of AUBURN.

SUMMARY:

The purpose of this component of the research project was to develop pertinent data on the use of Ulva (seaweed) meal *Ulva pertussa* as a feed ingredient/protein source in practical diets for Pacific white shrimp, *Litopenaeus vannamei*. To help understand variation in production, the report provides nutrient composition of both pooled and individual samples. Pooled samples of three Ulva meals were included in a digestibility trial to compare digestibility with other common ingredients. In all trials the basal diet was formulated to contain 35% crude protein and 8% lipid. In the first trial incremental levels of fishmeal were replaced with Ulva meal (Batch 1) on an iso-nitrogenous basis. Results demonstrated a clear depressing in growth as fishmeal was replaced. In the second trial, fishmeal levels were fixed and soybean meal was replaced using incremental level of *Ulva* meal from the second batch. Two additional diets were formulated to allow comparison of high inclusion levels of all three batches. Results confirm reductions in performance as *Ulva* meal is increased - particularly at high levels of inclusion. These data

showed significant difference between batches of *Ulva*, with the second batch (Batch 2) producing the poorest results. It is known from the literature that *Ulva* contains a number of biologically active compounds, and secondly, that it can be used at low levels of inclusion with potential benefits of these compounds. However, it was critical that we understand upper limits for inclusion of the Ulva meal, and possible reasons for the poor performance obtained in the first trials. To further elucidate the earlier poor growth results, and study the possibility that a digestible protein might have limited the observed poor shrimp growth, a third trial was initiated for which feeds were formulated on a digestible protein basis. Since methionine and lysine are two most limiting amino acids in shrimp feeds, these amino acids were also supplemented. In this follow-on trial, growth and survival were both significantly reduced as the level of Ulva meal (Batch 2) was increased. Although, growth and survival were both depressed, magnitudes were less than those of the previous trials indicating that protein quality may be only part of the problem, and other non-protein components of the *Ulva* meal could be implicated as causal of the poor growth performance observed. To survey possible problems caused by high levels of minerals the meals and select diets were analyzed for mineral content. Clearly there are shifts in mineral profiles; however, there is no obvious correlation to a mineral and this research team feels that it is unlikely a mineral toxicity. To continue further to possibly elucidate other possible causal factors, is, unfortunately, beyond the scope of this BARD-funded research, but if investigated would include investigating possible anti-nutrients present in the *Ulva* meals. Leading us to the conclusion, that if *Ulva* meals are to be use to their full potential as fishmeal replacements in shrimp diets, the anti-nutritional components will need to be specifically and unambiguously identified and verified in a plethora of *Ulva* species and cultured batches of the alga. In such follow-on research, hopefully specific species or strains of *Ulva* seaweeds, together with specific culture protocols limiting build-up of anti-nutritional compounds in the cultured biomass could be developed, and/or specific pre-formulation processing technologies that would remove, and/or significantly reduce the anti-nutritional compounds present in the cultured, raw meals, in order to consistently produce a high quality commercial fishmeal replacement product from cultured marine plants for inclusion in more renewably produced shrimp diets.

Materials and Methods – Marine Shrimp Nutrition and Diet Formulations

Experimental diets

Primary ingredients and four pooled batches of sun dried *Ulva pertussa* meal were analyzed for proximate composition, amino acids and minerals (Table 1 and 2) and the diets were formulated. Additionally, prior to pooling batch two, the 7 individual daily collections of *Ulva* meal were sampled and analyzed individually (Table 3). Upon completion of analysis diets were made by weighing pre-grounding dry ingredients and oil which was then mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air-dried (< 45 °C) to a moisture content of 8-10%. Pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed.

In trial 1, 2, 3 and 4 diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). In most diets substitution was done on a protein to protein basis however in trial 3, ingredient replacement was done on a digestible protein basis. In trial 1, five experimental diets were formulated to contain increasing levels (0, 6.35, 12.70, 19.05, and 25.40%) of the first batch of *Ulva* meal (UM1) as a replacement for fish meal (Table 3a). In trial 2, nine experimental diets were formulated (Table 4a). The basal diet for this and subsequent trials was designed to have 60 g/kg (6%) fishmeal in all formulations to help stabilize nutrients as well as palatability. The first seven diets utilized increasing levels of the second batch of *Ulva* meal (UM2) (0, 5, 10, 15, 20, 25, and 30%) to replace soybean meal. Diet 8 and Diet 9 utilized high incorporation of Ulva meal from the first and third batch, respectively. This allowed a comparison of all three meals at equivalent levels of protein replacement. In trial 3, four experimental diets were formulated using the first three batches of *Ulva* meal replacing soybean meal on a digestible protein basis. (Table 6a). Digestibility values for the meals were determined using standard methods and 1% chromic oxide as an inert marker (Table 8a). Test diets were made on a dry matter basis using a 70:30 mixture of the reference diet and test ingredients.

The ingredients (Table 1) were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition (g 100 g^{-1} as is) and amino acid profile (g 100 g^{-1} as is) and mineral profiles (Table 2) by the Soil Lab

(Auburn, AL, USA). Daily collections of UM2 samples (Table 3) collected across seven different dates were analyzed individually at Midwest laboratories (Omaha, NE, USA) for proximate and mineral composition. Diets (Table 4a, Table 5a, Table 6a, and Table 7a) were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition (g 100 g^{-1} as is) and amino acid profile (g 100 g^{-1} as is) and Soil Lab (Auburn, AL, USA) for mineral composition (mg kg^{-1}).

Growth trials

The trial 1 utilized 5 treatments with 7 replicates in each treatment. It was conducted in a semi-closed recirculation system. Juvenile shrimp were obtained from the nursery system and selected by hand-sorting to a uniform size. Juvenile shrimp (initial weight 0.26 ± 0.02 g) were stocked into 35 tanks with 10 shrimp in each aquarium (80L). A sub-sample of shrimp from the initial stocking was retained for whole body analysis to be utilized for later protein retention analysis. As shrimp are difficult to handle, intermittent weights were not taken. However, shrimp were counted to readjust daily feed input on a weekly basis. Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-0.9 g $week^{-1}$. Therefor for ten shrimp in a given tank, a fixed ration of 0.67 g day^{-1} for the first week, 1.45 g day^{-1} for the second week, 2.06 g day^{-1} for the third week, and 2.31 g day^{-1} for the fourth week, 2.57 g day^{-1} for the fifth week, and 2.83 g day^{-1} for the six week was offered over 4 feedings.

The trial 2 was conducted in the same semi-closed recirculation system which is mentioned above. It utilized 9 treatments with 4 replicates in each treatment. Juvenile shrimp (initial weight 0.24 ± 0.01 g) were stocked into 36 tanks with 10 shrimp in each aquarium (80L). A sub-sample of shrimp from the initial stocking was retained for whole body samples to be utilized for later protein and phosphorus retention analysis. Shrimp were counted to readjust daily feed input on a weekly basis. Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-0.9 g $week^{-1}$ thereafter. Consequently, for each tank a fixed ration of 0.62 g day^{-1} for the first week, 1.23 g day^{-1} for the second week, 2.06 g day^{-1} for the third and fourth week, and 2.31 g day^{-1} for the fifth week was offered over 4 feedings.

The trial 3 was conducted in the same semi-closed recirculation system which is mentioned above. It utilized 4 treatments with 4 replicates in each treatment. Juvenile shrimp (initial weight 0.98 ± 0.01 g) were stocked into 16 tanks with 10 shrimp in each aquarium (80L). A sub-sample of shrimp from the initial stocking was retained for whole body samples to be utilized for later protein and phosphorus retention analysis. As shrimp are difficult to handle, intermittent weights were not taken. However, shrimp were counted to readjust daily feed input on a weekly basis. Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-1.3 g $week^{-1}$ thereafter. Consequently, for each tank of 10 shrimp a fixed ration of 2.1 g day^{-1} for the first week, 2.3 g day^{-1} for the second week, 2.8 g day^{-1} for the third week, 3.1 g day^{-1} for the fourth week, 3.4 g day^{-1} for the fifth week, and 3.7 g day^{-1} for the sixth week was offered over 4 feedings.

The trial 4 was conducted in a similar semi-closed recirculation system to what was previously described. It utilized 5 treatments with 4 replicates in each treatment. Juvenile shrimp (initial weight 0.15 ± 0.01 g) were stocked into 20 tanks with 10 shrimp in each aquarium (80L). Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-1.3 g $week^{-1}$ thereafter. Consequently, for each tank of 10 shrimp a fixed ration of 2.1 g day^{-1} for the first week, 2.3 g day^{-1} for the second week, 2.8 g day^{-1} for the third week, 3.1 g day^{-1} for the fourth week, 3.4 g day^{-1} for the fifth week, and 3.7 g day^{-1} for the sixth week was offered over 4 feedings.

At the conclusion of each growth trials, shrimp were counted and group weighted. Mean final weight, feed conversion ratio (FCR), weight gain (WG), biomass, and survival were determined (Table 4c, 5d, 6c, 7c). After obtaining the final total weight of shrimps in each aquarium, 4 shrimps were randomly selected and frozen at -20 °C for subsequent determination of whole body composition. Proximate composition (Table 4d, 5e, 6d, 7d) of whole shrimp was analyzed by Midwest Laboratories, Inc. (Omaha, NE, USA) or University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA). Mineral profiles of whole shrimp and select algae meals were analyzed by Soils lab (Auburn, AL, USA). Feed conversion

ratio (FCR) and apparent net protein retention (PR) were calculated using the following equations:

FCR = feed offered per shrimp/mean weight gain;

Protein retention Protein retention (%) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.

Water quality monitoring

Dissolved oxygen (DO), temperature, and salinity were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). The pH was measured twice weekly by using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Water samples were taken to measure total ammonia-nitrogen (TAN) and nitrite every week. TAN and nitrite were determined by the methods described by (Solorzano, 1969) and (Spotte, 1979), respectively. In trial 1, DO, temperature, salinity, pH, TAN, and nitrite were maintained within acceptable ranges for *L. vannamei* at 6.19 ± 0.25 mg L^{-1}, 28.4 ± 0.8 °C, 11.8 ± 0.4 ppt, 7.23 ± 0.22, 0.079 ± 0.041 mg L^{-1}, and 0.039 ± 0.021 mg L^{-1}, respectively. In trial 2, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 5.82 ± 0.26 mg L^{-1}, 29.7 ± 0.8 °C, 8.6 ± 0.4 ppt, 7.5 ± 0.5, 0.052 ± 0.107 mg L^{-1}, and 0.003 ± 0.004 mg L^{-1}, respectively. In trial 3, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.20 ± 0.72 mg L^{-1}, 29.5 ± 0.9 °C, 8.4 ± 1.0 ppt, 7.5 ± 0.3, 0.092 ± 0.103 mg L^{-1}, and 0.050 ± 0.039 mg L^{-1}, respectively. Water quality conditions in all the trials were suitable for normal growth and survival of this species.

Digestibility trial

The digestibility trial was conducted in the mentioned recirculation system and utilized 6 shrimp per aquaria with 6 aquaria per dietary treatment. Once acclimated for 3 days to the test diets, feces from two aquaria were pooled (n=3) and collected over a 5 day period or until adequate samples were obtained. To obtain fecal samples, the aquaria were cleaned by siphoning before each feeding with the first collection of the day discarded. After the aquaria were cleaned, the shrimp were offered an excess of feed and then about 1 hour later feed was removed and feces were collected by siphoning onto a 500μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight was obtained, and then stored in freezer (-

20 °C) until analyzed. Apparent digestibility coefficient for dry matter, protein, energy and amino acids were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. Chromium concentrations were determined by the method of (McGinnis, Kasting, 1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540nm. Gross energy of diets and fecal samples were analyzed with a Semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Protein were determined by micro-Kjeldahl analysis (Ma, Zuazaga, 1942). The apparent digestibility coefficient of dry matter (ADMD), protein (ADP), energy (ADE) and amino acids (ADAA) were calculated according to (Cho, Slinger, Bayley, 1982)as follows:
ADMD (%)= 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces)]; ADP, ADE, and ADAA (%)=100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces) × (% nutrient in feces / % nutrient in feed).

The apparent digestibility coefficients (ADC) of the test ingredients for dry matter, energy, protein and amino acids were calculated according to Bureau, Hua, Cho (2006) as follows:

$$ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]$$

where D_{ref} = % nutrient (or KJ/g gross energy) of reference diet mash (as is); D_{ingr} = % nutrient (or KJ/g gross energy) of test ingredient (as is).

Statistical analysis

All the data were analyzed using SAS software tools (V9.3. SAS Institute, Cary, NC, USA). Data from the growth were analyzed using one-way analysis of variance (ANOVA) to determine significant differences (P<0.05) among treatments followed by the Tukey's multiple comparison test to determine difference between treatments.

Results

The purpose of this component of the research project was to develop pertinent data on the use of *Ulva* meal *Ulva pertussa* as a feed ingredient or protein source in practical diets for Pacific white shrimp, *Litopenaeus vannamei*. The reported trials were conducted at the Aquatic Animal Nutritional Laboratory, School of Fisheries, Aquaculture, and Aquatic Sciences (Auburn, AL,

USA). The basal diet (35% crude protein, 8% lipid) used in the trials was primarily composed of fish meal, soybean meal, corn protein concentrate, whole wheat and corn starch. In trial 1, five diets contain increasing levels (0, 6.35, 12.70, 19.05, and 25.40%) of *Ulva* meal (UM1) replacing fish meal were developed (Table 4). A six-week growth trial was completed using juvenile shrimp (initial weight: 0.26g) stocked at density of 10 shrimp per tank over 6 week feeding periods.

The shrimp were cultured in a semi-closed recirculating system containing a biological filter, bead filter reservoir, supplemental aeration and a circulation pump. At the end of the growth trial, significant reductions of biomass, final mean weight, weight gain, and protein retention and increased feed conversion ratio (FCR) were observed when UM1 was supplemented at 12.7, 19.05, and 25.4% of the diets (Table 4c). With regards to the proximate composition of shrimp body, lipid content was significantly reduced when *Ulva* meal was supplemented in the diets (Table 4d). However, no significant difference was observed with regards to the protein content of whole body.

As the replacement of fishmeal results in shifts in numerous nutrient as well as possible palatability changes of the diet in we chose to shift the nutrition model to replace soybean meal in the later trials as this results in fewer shifts in nutrients. In trial 2, the first seven diets utilized increasing levels of *Ulva* meal originating from the second batch (UM2 at 0, 5, 10, 15, 20, 25, and 30%) and was used as a replacement for soybean meal on an iso-nitrogenous basis (Table 5a). Diet 8 and Diet 9 (T_2D8 and T_2D9, respectively) utilized high levels of *Ulva* meal from the first and second batch to replace soybean meal comparable to that of UM2 (Diet 5). Thus allowing for a comparison of all three batches of meals.

This 5-week growth trial was completed using juvenile shrimp (initial weight: 0.24g) stocked at density of 10 shrimp per tank. The shrimp were cultured in the same semi-intensive recirculating system as trial 1. At the end of the growth trial, significantly reductions of biomass, WG, final mean weight as well as survival were observed along with increased FCR (Table 5d). Shrimp fed with UM1 (Diet 9) and UM3 (Diet 8) replacing the same levels of soybean meal as UM2 (diet 5) exhibited higher WG and lower FCR clearly demonstrating differences across batches. Although

UM1 produced the poorest results, UM2 and UM3 also resulted in significant reductions in growth and increases in FCR.

The poor response of increasing levels of *Ulva* meal in the diet could be due to a range of dietary problems with increasing levels as well as problems with nutrient shifts between batches of algae. One hypothesis is that due to the high ash content of the *Ulva* meals that there may be a mineral toxicity occurring. Hence the mineral profiles of the first three meals (Table 2) and the diets from the second growth trial (Table 5c). Clearly there are shifts in mineral profiles with a number of minerals increasing in levels. However, if one also assumes that diets made with UM1 would have higher levels than UM2 and 3 there is no obvious correlation to a mineral that could be causing a toxicity. Some sort of mineral toxicity could be responsible the authors feel this is unlikely. Other possible nutritional problems could come about by limitations of protein, amino acids or digestibly of the protein and amino acids. This can be mediated by formulating on a digestibility basis and supplementing possible limiting amino acids.

In addition to the growth trial the ingredient was included in a larger digestibility trial. The results of several ingredients are included as a reference. The protein, energy, and amino acids digestibility of the ingredient were low compared to the fish meal and soybean meal (Table 8b and Table 8c), which might serve as a partial explanation for the reduced growth. To elucidate if digestible protein was limiting growth, a third trial was initiated for which feeds were formulated on a digestible protein basis. Since methionine and lysine are typically the two most limiting amino acids in shrimp feeds, they are also balanced on the digestible basis.

In trial 3, four diets utilized high levels of *Ulva* meal from three batches to replace soybean meal on the digestible protein basis. The 6-week growth trial was completed using juvenile shrimp (initial weight: 0.98g) stocked at density of 10 shrimp per tank. The shrimp were cultured in the same semi-intensive recirculating system as trial 1 and trial 2. At the end of the growth trial, growth and survival were not affected when experimental diets supplemented with first batch Ulva, however, significantly reduced growth and survival were detected when shrimp fed with diets contain second and third batch *Ulva*. The same response was observed in trial 2, in which reduced growth the survival was also observed for shrimp offered diets with UM2. This

indicated there might be some other factors affecting the growth of shrimp. The limited reduction in performance of shrimp offered high levels of UM1 and UM3 indicate that part of the problem is probably due to low digestibility. However, this did not solve the problem for UM2 which had both poor survival and poor growth.

If one looks at this from a feed manufacturing side, an ingredient is only going to be used if it can be included in a feed formulation at a significant rate or it brings special properties to the diets. Looking across the diets that have been evaluated there is no indication of a benefit of *Ulva* meal supplementation at low levels and there is a major reduction in performance when 20-25% is included in the diets. On an iso-nitrogenous basis the high levels of inclusion evaluated in this study (20-25% of the diet) we are only brining in around 5.5% protein or on a digestible protein basis 3% protein. Given the reduction of growth that is occurring across the growth trial one would have to conclude that it is not protein quality but some other component of the meal. One theory that has been advanced but is beyond the scope of this research is that *Ulva* is producing a chemical defense against herbivory.

Comparison to Literature

There are relatively few studies looking at the efficacy of *Ulva* meal in aquatic animal feeds particularly with regards to shrimp. A number of these publications approached the use of *Ulva* meal as a dietary supplement with biologically active compounds for which animal performance may be improved due to a range of factors. In general, a number of studies demonstrate that low levels (≤5% of the diet) generally did not result in poor performance in both freshwater (e.g. tilapia, (Natify, Droussi, Berday, Araba, Benabid, 2015), (Guroy, Cirik, Guroy, Sanver, Tekinay, 2007); African catfish, (Abdel-Warith, Younis el, Al-Asgah, 2016) or in marine fish such as gilthead seabream, (Emre, Ergun, Kurtoglu, Guroy, Guroy, 2013). In a few cases improvements in growth have been reported. For example, Ergun et al 2009 reported that 5% *Ulva* meal incorporated into diets for *Oreochromis niloticus* resulted in improved performance. Cruz-SuÁRez, Tapia-Salazar, Nieto-LÓPez, Guajardo-Barbosa, Ricque-Marie (2009) reported improvements in the Pacific white shrimp growth when *Ulva* meal was included at 3.3% of the diet.

However, when higher levels of *Ulva* meal were evaluated most of the aforementioned authors identified significant depressions in performance of the fish and shrimp (Serrano, Santizo, 2014). It should also be noted that that Harbor Branch had evaluated fresh *Ulva* as a food for shrimp and also found poor performance at high levels indicating that this may not be a processing problem (personal communication Dr. Laramore).

These results are in agreement with those observed in the shrimp in that low levels of inclusion did not significantly influence the performance of the shrimp and that as the levels were increased there was a negative dose response to increased levels of inclusion. Based on the results of our studies as well as those of quality publications with both fish and shrimp it is clear the *Ulva* meals can be utilized at low levels but high levels of inclusion are problematic across numerous species. The present results would indicate that this is likely due to the presence of an anti-nutrient which should be identified. Given the potential of *Ulva* meal to trap nutrients, developing strains of *Ulva* with improved nutritional profiles should be a goal of future studies.

Table 1. Proximate composition, phosphorus content, and amino acid profile of the primary protein sources used in diet formulations.

Composition[1] (As is %)	Ulva meal 1	Ulva meal 2	Ulva meal 3	Ulva meal 4	Menhaden fish meal	Soybean meal
Crude Protein	20.64	27.24	26.80	38.16	62.78	44.89
Moisture	8.89	13.74	11.19	8.41	7.99	10.97
Crude Fat	0.53	0.12	0.42	0.10	10.56	3.78
Crude Fiber	5.17	2.93	4.07	5.57	0.00	3.20
Ash	46.01	22.18	20.31	13.49	18.75	6.67
Phosphorus	0.43	0.30	-	0.42	3.15	0.66
Alanine	1.64	2.03	1.89	2.68	3.91	2.04
Arginine	0.99	1.39	1.01	1.77	3.68	3.35
Aspartic Acid	2.12	2.67	3.23	3.46	5.34	5.1
Cysteine	0.34	0.39	0.46	0.49	0.47	0.62
Glutamic Acid	2.02	2.59	3.02	3.35	7.47	8.24
Glycine	1.17	1.59	1.29	2	4.88	2.04

Histidine	0.25	0.40	0.22	0.45	1.63	1.2
Hydroxylysine	0.17	0.12	0.10	0.21	0.2	0.05
Hydroxyproline	0.2	0.30	0.38	0.35	1.03	0.05
Isoleucine	0.8	1.06	0.92	1.39	2.42	2.17
Leucine	1.22	1.87	1.50	2.43	4.21	3.57
Lysine	0.95	1.22	0.82	1.51	4.67	3.06
Methionine	0.26	0.44	0.46	0.63	1.61	0.66
Ornithine	0.02	0.02	0.02	0.04	0.07	0.03
Phenylalanine	0.98	1.37	1.16	1.78	2.39	2.35
Proline	0.76	1.17	1.02	1.5	3.08	2.39
Serine	0.91	1.05	0.93	1.47	2.11	1.90
Taurine	0.15	0.18	0.18	0.18	0.73	0.13
Threonine	0.94	1.17	1.13	1.56	2.41	1.75
Tryptophan	0.16	0.20	0.22	0.266	0.62	0.62
Tyrosine	0.48	0.77	0.49	0.94	1.67	1.64
Valine	1.17	1.56	1.40	2.13	2.99	2.34

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 2. Mineral composition of the three batches *Ulva* meal (UM1, UM2, and UM3) used to formulate feeds.

Mineral composition mg kg^{-1}	Ulva meal1	Ulva meal2	Ulva meal3
As	1.6	1.3	0.6
B	76.2	38.8	70.0
Ba	13.8	2.6	1.6
Cd	50.4	8.3	9.6
Co	3.0	0.8	0.6
Cr	9.7	1.8	0.7
Cu	26.5	17.5	56.9
Mn	112.4	21.1	17.8
Ni	7.7	2.1	3.3
Pb	10.8	2.0	1.2
Se	5.3	3.9	2.8
Si	70.3	68.4	16.4
Zn	63.1	34.6	18.9
Zr	1.0	1.0	1.0
Al	4173.2	380.5	31.7
Fe	9086.7	581.6	70.0
Ca	22923.1	4855.3	3604.3
K	19853.7	22073.4	38982.8
Mg	25678.6	29245.3	11120.9
Na	47936.2	16305.7	28187.4
P	3963.4	3208.5	3080.8
S	34546.9	45351.5	29552.4

Table 3. Proximate and mineral composition of *Ulva* meal (Batch 2) collected from 7 different dates.

Parameter (As is basis %)[1]	Dates (2015)				
	7/21	7/30	8/16	8/20	8/23
Moisture	83.99	87.14	85.59	82.78	83.32
Crude protein	28.2	19.4	29	28.3	27.3
Crude fat	0.46	n.d.	0.2	n.d.	n.d.
Fiber	10	13.9	13.4	10.9	11.3
Ash	17.3	39.2	19.8	15.6	17.1
Sulfur	4.38	3.64	3.78	4.19	4.16
Phosphorus	0.32	0.37	0.38	0.35	0.31
Potassium	2.71	2.26	1.82	2.2	1.95
Magnesium	3.12	3.07	3.00	3.25	3.21
Calcium	0.42	2.01	0.86	0.47	0.44
Sodium	1.26	2.74	1.46	0.89	1.55
Iron (ppm)	331	6780	2040	424	450
Manganese (ppm)	21.1	99.2	47.0	22.9	22.2
Copper (ppm)	7.7	28.2	11.0	7.8	7.6
Zinc (ppm)	37.6	79.3	64.0	49.0	38.4

[1]Analyses conducted by Midwest Laboratories.

n.d.: not detected.

Table 4a. Formulation of test diets designed to evaluate *Ulva* meal as a replacement for fish meal on an iso-nitrogenous basis in juvenile shrimp (Trial 1).

Ingredient (g kg^{-1} As is basis)	T_1D_1	T_1D_2	T_1D_3	T_1D_4	T_1D_5
Menhaden fish meal[1]	100.0	80.0	60.0	40.0	20.0
Soybean meal[2]	487.0	487.0	487.0	487.0	487.0
Corn protein concentrate[3]	80.0	80.0	80.0	80.0	80.0
Ulva meal	0.0	63.5	127.0	190.5	254.0
Menhaden Fish Oil[2]	56.5	57.5	58.6	59.7	60.7
Trace Mineral premix[5]	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	16.2	19.0	21.5	24.0	26.5
Lecithin[9]	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5
Corn Starch[4]	225.4	177.8	130.5	83.1	35.8

[1] Omega Protein Inc., Huston TX, USA.

[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

[4] MP Biomedicals Inc., Solon, Ohio, USA.

[5]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.

[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.

[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.

[9] The Solae Company, St. Louis, MO, USA.

Table 4b. Proximate composition (g/100g as is) and amino acid profile (g/100g as is) of the test diets used in the fish meal replacement growth trial (Trial 1).

Composition[1] (As is %)	T_1D_1	T_1D_2	T_1D_3	T_1D_4	T_1D_5
Ulva levels (%)	0	6.35	12.7	19.05	25.4
Crude Protein	36.83	36.52	36.60	36.28	35.65
Moisture	5.46	6.56	5.12	7.15	8.70
Crude Fat	10.09	8.94	9.06	8.22	7.51
Crude Fiber	2.92	3.08	3.48	3.22	3.33
Ash	6.54	8.92	11.80	14.40	16.58
Alanine	2.03	2.00	2.08	2.08	2.04
Arginine	2.24	2.21	2.23	2.19	2.14
Aspartic Acid	3.56	3.53	3.62	3.58	3.53
Cysteine	0.48	0.47	0.48	0.49	0.49
Glutamic Acid	6.39	6.18	6.32	6.11	6.01
Glycine	1.65	1.63	1.63	1.61	1.55
Histidine	0.92	0.89	0.89	0.85	0.82
Hydroxylysine	0.04	0.06	0.08	0.09	0.06
Hydroxyproline	0.13	0.12	0.11	0.10	0.09
Isoleucine	1.68	1.64	1.70	1.68	1.6
Lanthionine	0	0	0	0	0
Leucine	3.43	3.29	3.41	3.34	3.21
Lysine	2.13	2.08	2.06	1.99	1.91
Methionine	0.66	0.64	0.63	0.62	0.63
Ornithine	0.02	0.02	0.02	0.02	0.02
Phenylalanine	1.85	1.86	1.94	1.92	1.81
Proline	2.09	2.08	2.12	2.09	1.98
Serine	1.51	1.50	1.53	1.47	1.51
Taurine	0.18	0.17	0.16	0.14	0.13
Threonine	1.36	1.35	1.38	1.37	1.35
Tryptophan	0.42	0.39	0.39	0.39	0.37
Tyrosine	1.45	1.44	1.49	1.47	1.39
Valine	1.78	1.77	1.82	1.80	1.76

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 4c. Protein retention efficiency and growth performance of juvenile Pacific white shrimp (0.26±0.02g) offered diets with different *Ulva pertussa* levels (0, 6.35, 12.70, 19.05, and 25.40%) as a fish meal replacement over a six-week growth trial (Trial 1).

Diet	Ulva Levels (%)	Final Biomass (g)	Final Mean Weight (g)	WG[3] (%)	FCR[2]	Survival (%)	PRE[4] (%)
T_1D_1	0	44.63[a]	5.01[a]	1792.8[a]	1.83[b]	88.6	25.70[ab]
T_1D_2	6.35	45.45[a]	5.09[a]	1830.9[a]	1.81[b]	88.6	27.16[a]
T_1D_3	12.70	39.58[ab]	4.30[ab]	1555.1[b]	2.15[ab]	91.4	23.07[ab]
T_1D_4	19.05	36.10[ab]	3.88[b]	1389.1[b]	2.36[a]	92.9	20.20[b]
T_1D_5	25.40	32.26[b]	3.87[b]	1407.4[b]	2.43[a]	82.9	20.36[b]
P-value		0.0175	0.0006	0.0003	0.0039	0.2451	0.0073
PSE[1]		1.1253	0.0868	28.9568	0.0491	1.2074	0.5699

[1] Pooled standard error.

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

[3] WG: Weight gain = (Final weight-initial weight)/initial weight*100%.

[4] PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 4d. Proximate analysis of whole shrimp body offered varying *Ulva pertussa* levels (0, 6.35, 12.70, 19.05, and 25.40%) as a iso-nitrogenous replacement of fishmeal over a six week growth trial (Trial 1).

Diet	Ulva Levels (%)	Moisture (%)	Crude protein (%)	Crude lipid (%)
T_1D_1	0	76.88	72.77	8.04[a]
T_1D_2	6.35	76.29	73.63	6.12[b]
T_1D_3	12.70	76.99	74.27	5.73[b]
T_1D_4	19.05	76.37	72.83	5.99[b]
T_1D_5	25.40	76.83	74.11	5.09[b]
P-value		0.7933	0.2576	0.0006
PSE[1]		0.1340	0.2240	0.1613

[1] Pooled standard error.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 5a. Formulation of test diets designed to evaluate *Ulva* meal as a replacement for soybean meal on an iso-nitrogenous basis in juvenile shrimp (Trial 2).

Ingredient (g kg^{-1} As is basis)	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9
Menhaden fish meal[1]	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Soybean meal[2]	555.5	525.5	496.0	466.0	437.5	408.0	378.0	437.5	437.5
Corn protein concentrate[3]	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Ulva meal 2	0.0	50.0	100.0	150.0	200.0	250.0	300.0		
Ulva meal 1								236.0	
Ulva meal 3									263.0
Menhaden Fish Oil[2]	58.4	58.8	59.2	59.6	60.0	60.4	60.8	60.0	58.9
Trace Mineral premix[5]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	18.5	18.5	19.0	19.0	19.5	19.5	20.0	19.0	17.0
Lecithin[9]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Methionine	0.7	0.6	0.6	0.6	0.5	0.5	0.4	0.3	0.7
Corn Starch[4]	150.4	130.1	108.7	88.3	66.0	45.1	24.3	30.7	6.4

[1] Omega Protein Inc., Huston TX, USA.

[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

[4] MP Biomedicals Inc., Solon, Ohio, USA.

[5] Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.

[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin,

0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.

[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.

[9] The Solae Company, St. Louis, MO, USA.

Table 5b. Proximate composition (g/100g as is), phosphorus content, and amino acid profile (g/100g as is) of the test diets used in the soybean meal replacement growth trial (Trial 2).

Composition[1] (As is %)	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9
Ulva levels (%)	0	5	10	15	20	25	30	23.6	26.3
Crude Protein	36.46	36.67	35.91	36.78	36.64	36.69	37.46	37.08	36.34
Moisture	7.32	7.92	9.44	7.56	8.29	8.03	6.46	7.74	8.48
Crude Fat	10.02	8.49	8.68	9.71	8.90	8.28	6.29	6.35	7.01
Crude Fiber	3.54	3.43	3.65	3.64	3.79	4.10	4.09	3.86	3.86
Ash	6.49	7.61	8.47	9.02	10.17	10.47	11.48	10.56	16.77
Phosphorus	0.99	1.01	1.00	1.03	1.01	1.06	1.02	1.00	1.02
Alanine	1.86	1.90	1.91	2.00	2.11	2.05	2.18	2.03	1.99
Arginine	2.30	2.26	2.20	2.22	2.19	2.18	2.24	2.12	2.12
Aspartic Acid	3.68	3.62	3.56	3.62	3.61	3.60	3.68	3.72	3.50
Cysteine	0.50	0.47	0.47	0.48	0.48	0.47	0.47	0.50	0.47
Glutamic Acid	6.68	6.48	6.25	6.29	6.26	5.98	6.07	6.14	5.95
Glycine	1.64	1.68	1.67	1.69	1.84	1.77	1.86	1.73	1.62
Histidine	0.91	0.88	0.85	0.86	0.84	0.82	0.84	0.79	0.80
Hydroxylysine	0.05	0.05	0.05	0.06	0.07	0.07	0.07	0.06	0.07
Hydroxyproline	0.11	0.10	0.11	0.12	0.18	0.37	0.16	0.22	0.17
Isoleucine	1.64	1.60	1.56	1.60	1.58	1.57	1.61	1.56	1.54
Leucine	3.22	3.14	3.09	3.16	3.20	3.08	3.22	3.03	3.00
Lysine	2.04	1.99	1.95	1.95	1.91	1.92	1.94	1.86	1.86
Methionine	0.70	0.67	0.66	0.67	0.68	0.66	0.67	0.63	0.61
Ornithine	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Phenylalanine	1.85	1.82	1.80	1.84	1.86	1.82	1.89	1.79	1.76
Proline	2.09	1.97	2.06	1.96	2.13	2.02	1.99	2.03	2.00
Serine	1.53	1.53	1.47	1.52	1.51	1.49	1.54	1.45	1.46
Taurine	0.14	0.16	0.15	0.15	0.15	0.16	0.17	0.17	0.14
Threonine	1.35	1.35	1.34	1.37	1.38	1.39	1.44	1.37	1.34
Tryptophan	0.50	0.50	0.47	0.48	0.48	0.47	0.49	0.44	0.45
Tyrosine	1.19	1.19	1.14	1.21	1.19	1.17	1.21	1.10	1.13
Valine	1.83	1.81	1.80	1.88	1.88	1.86	1.91	1.84	1.80

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 5c. Mineral profile (mg kg^{-1} as is) of the test diets used in the growth trial 2.

	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5	T_3D_6	T_3
Ulva levels (%)	0	5	10	15	20	25	3
As	0.7	0.6	0.4	0.6	0.8	0.9	0
B	17.6	18.6	19.3	20.5	21.5	21.5	23
Ba	5.1	5.3	5.7	5.1	5.0	4.5	4
Cd	4.5	0.9	13.2	1.2	14.3	12.6	6
Co	1.1	1.3	1.2	1.2	1.2	1.0	1
Cr	1.0	1.0	1.1	1.1	1.1	1.1	1
Cu	39.4	110.3	21.0	22.9	18.6	23.9	27
Mn	34.2	35.4	34.5	33.6	33.5	32.7	34
Mo	3.9	4.0	3.4	3.3	3.5	2.5	2
Ni	3.1	3.2	3.1	2.6	2.7	2.4	2
Pb	1.0	1.3	1.7	0.5	1.4	0.5	0
Se	3.3	4.6	2.3	3.1	3.8	4.4	4
Si	57.9	81.0	107.0	120.8	119.9	131.7	13
Zn	158.1	165.1	145.8	145.8	135.6	155.4	15
Zr	0.9	1.0	0.9	0.9	1.0	0.9	0
Al	97.8	119.5	133.1	160.8	173.8	185.6	19
Fe	59.2	43.8	69.4	74.6	66.9	74.4	66
Ca	8526.1	9742.0	9005.7	9466.3	9109.3	8946.9	951
K	12713.5	13459.0	13697.7	14587.5	14869.2	14917.6	159
Mg	1945.1	3398.6	4679.4	6190.1	7468.4	8664.7	959
Na	834.0	1691.1	2439.2	3312.9	4134.3	4763.4	571
P	10260.7	10783.9	10192.6	10654.6	10338.0	9943.0	103
S	3797.7	5854.7	7652.0	10011.1	11811.5	13583.8	161

Table 5d. Protein retention efficiency and growth performance of juvenile Pacific white shrimp (0.24±0.01g) offered diets with different *Ulva pertussa* levels in the soybean meal replacement growth trial for five weeks (Trial 2).

Diet	Ulva levels (%)	Final Biomass (g)	Final Mean Weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_2D_1	0	43.31^{a}	4.55^{a}	1734.21^{a}	1.46^{b}	95.0^{a}
T_2D_2	5	36.19^{ab}	3.70^{ab}	1398.22^{ab}	1.83^{ab}	97.5^{a}
T_2D_3	10	28.40^{bc}	3.25^{ab}	1241.46^{ab}	2.23^{ab}	87.5^{ab}
T_2D_4	15	23.89^{cd}	2.58^{b}	948.74^{b}	2.82^{ab}	92.5^{a}
T_2D_5	20	18.98^{cd}	2.53^{b}	990.26^{b}	2.96^{ab}	75.0^{ab}
T_2D_6	25	16.34^{cd}	2.56^{b}	943.46^{b}	3.53^{a}	67.5^{b}
T_2D_7	30	15.50^{d}	2.45^{b}	864.67^{b}	3.37^{ab}	65.0^{b}
T_2D_8	23.6	26.14^{bcd}	2.96^{b}	1131.07^{b}	2.61^{ab}	87.5^{ab}
T_2D_9	26.3	27.10^{bcd}	3.09^{b}	1226.92^{ab}	2.36^{ab}	87.5^{ab}
P-value		<0.0001	0.0002	0.0008	0.0201	0.0006
PSE[1]		1.2872	0.1392	61.8604	0.2020	2.6131

[1] Pooled standard error.

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

[3] WG: Weight gain = (Final weight-initial weight)/initial weight*100%.

[4] PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test

Table 5e. Proximate analysis of whole shrimp body offered varying *Ulva pertussa* levels diets in soybean meal replacement trial for five weeks (trial 2).

Diet	Ulva Levels (%)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude fiber	Ash
T_2D_1	0					
T_2D_2	5					
T_2D_3	10					
T_2D_4	15					
T_2D_5	20					
T_2D_6	25					
T_2D_7	30					
T_2D_8	23.6					
T_2D_9	26.3					
P-value						
PSE[1]						

[1] Pooled standard error.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test

Table 6a. Formulation of test diets designed to evaluate *Ulva* meal as a replacement for soybean meal on a digestible protein basis in juvenile shrimp (Trial 3).

Ingredient (g kg^{-1} As is basis)	T_3D_1	T_3D_2	T_3D_3	T_3D_4
Menhaden fish meal[1]	60.0	60.0	60.0	60.0
Soybean meal[2]	530.0	499.0	463.0	463.0
Corn protein concentrate[3]	80.0	80.0	80.0	80.0
Ulva meal 2[10]		220.0		
Ulva meal 1[10]			250.0	
Ulva meal 3[10]				250.0
Menhaden Fish Oil[2]	59.2	58.5	59.8	59.1
Trace Mineral premix[5]	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	25.0	25.0	25.0	25.0
Lecithin[9]	10.0	10.0	10.0	10.0
Cholesterol[4]	0.8	0.8	0.8	0.8
Methionine	0.5	0.4	0.4	0.4
Lyisine	0.0	0.0	0.7	1.1
Corn Starch[4]	208.5	20.3	24.3	24.6

[1] Omega Protein Inc., Huston TX, USA.

[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

[4] MP Biomedicals Inc., Solon, Ohio, USA.

[5]Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.

[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.

[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.

[9] The Solae Company, St. Louis, MO, USA.

Table 6b. Proximate composition[1] (g $100g^{-1}$ as is), mineral composition[2] and amino acid profile[1] (g $100g^{-1}$ as is) of the test diets used in the growth trial 3.

Composition (As is g $100g^{-1}$)	T_3D_1	T_3D_2	T_3D_3	T_3D_4
Crude protein	36.33	38.40	39.66	39.13
Moisture	7.15	7.59	8.93	8.34
Crude fat	9.39	9.03	9.01	8.68
Crude fiber	3.21	3.84	4.42	4.13
Ash	6.86	15.93	11.44	11.22
Phosphorus	1.36	1.25	1.24	1.37
Sulfur	0.4	1.06	1.27	1.08
Potassium	1.33	1.73	1.65	2.13
Magnesium	0.18	0.76	0.86	0.52
Calcium	1.31	1.79	1.17	1.3
Sodium	0.1	1.16	0.51	0.77
Iron (ppm)	149	1240	286	169
Manganese (ppm)	40.1	71.6	39.1	40.1
Copper (ppm)	16.8	22.9	20.2	28.7
Zinc (ppm)	183	215	187	194
Alanine	1.87	2.15	2.24	2.14
Arginine	2.18	2.26	2.34	2.21
Aspartic Acid	3.44	3.66	3.78	3.79
Cysteine	0.48	0.49	0.50	0.51
Glutamic Acid	6.33	6.43	6.33	6.24
Glycine	1.56	1.69	1.82	1.68
Histidine	0.86	0.86	0.89	0.80
Isoleucine	1.60	1.70	1.71	1.65
Leucine	3.28	3.49	3.50	3.32
Lysine	2.01	2.03	2.16	2.05

Methionine	0.64	0.62	0.67	0.65
Phenylalanine	1.85	2.00	2.05	1.90
Proline	2.13	2.16	2.22	2.22
Serine	1.48	1.61	1.68	1.59
Taurine	0.16	0.13	0.14	0.16
Threonine	1.29	1.43	1.50	1.44
Tryptophan	0.47	0.48	0.45	0.44
Tyrosine	1.33	1.38	1.44	1.33
Valine	1.73	1.95	2.01	1.94

[1] Proximate composition and amino acid profiles of test diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).
[2] Mineral composition was tested at Midwest Laboratories (Omaha, NE, USA).

Table 6c. Performance of juvenile Pacific white shrimp *L. vannamei* (Initial weight 0.98g) offered diets formulated to partially replace soybean meal on a digestible protein basis with three different batches of *Ulva* meal (Trial 3).

Diet	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_3D_1	79.3[a]	8.4[a]	766.6[a]	1.64[b]	95.0[a]
T_3D_2	66.8[a]	7.8[a]	689.7[a]	1.87[b]	85.0[ab]
T_3D_3	30.6[c]	4.9[b]	397.3[b]	3.58[a]	62.5[c]
T_3D_4	57.3[b]	7.2[a]	618.1[a]	2.09[b]	80.0[b]
PSE[1]	1.7351	2.3457	18.1043	0.1167	1.5427
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight-initial weight)/initial weight*100%.
[4] PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 6d. Proximate composition of shrimp at the conclusion of a 6-week growth trial in which shrimp were offered diets formulated to partially replace soybean meal on a digestible protein basis with three different batches of Ulva meal (Trial 3).

Diet	Ulva Levels (%)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude fiber	Ash
T_3D_1						
T_3D_2						
T_3D_3						
T_3D_4						
P-value						
PSE[1]						

[1] Pooled standard error.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 7a Formulation of test diets designed to evaluate *Ulva* meal as a replacement for soybean meal and fish meal on a iso-nitrogen basis in juvenile shrimp (Trial 4).

Ingredient (g kg^{-1} As is basis)	T_4D_1	T_4D_2	T_4D_3	T_4D_4	T_4D_5
Menhaden fish meal[1]	60.00	60.00	60.00	30.00	0.00
Soybean meal[2]	530.00	430.00	330.00	530.00	530.00
Corn protein concentrate[3]	80.00	80.00	80.00	80.00	80.00
Ulva meal 4[10]	0.00	120.00	240.00	47.50	95.00
Menhaden Fish Oil[2]	59.20	60.50	61.80	61.90	64.50
Trace Mineral premix[5]	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	25.00	26.00	26.00	29.00	31.00
Lecithin[9]	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.8	0.8	0.8	0.8	0.8
Lyisine	0.00	1.10	2.20	0.70	1.30

Methionine	0.50	1.10	1.70	1.00	1.50
Corn Starch[4]	208.50	184.50	161.50	183.10	159.90

[1] Omega Protein Inc., Huston TX, USA.

[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.

[4] MP Biomedicals Inc., Solon, Ohio, USA.

[5]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.

[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.

[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.

[9] The Solae Company, St. Louis, MO, USA.

Table 7b. Proximate composition[1] (g $100g^{-1}$ as is), mineral composition[2] and amino acid profile[1] (g $100g^{-1}$ as is) of the test diets used in the growth trial 4.

Composition (As is g $100g^{-1}$)	T_4D_1	T_4D_2	T_4D_3	T_4D_4	T_4D_5
Crude protein					
Moisture					
Crude fat					
Crude fiber					
Ash					
Alanine					
Arginine					
Aspartic Acid					
Cysteine					
Glutamic Acid					
Glycine					
Histidine					
Isoleucine					
Leucine					
Lysine					
Methionine					
Phenylalanine					
Proline					
Serine					
Taurine					
Threonine					
Tryptophan					
Tyrosine					
Valine					

[1] Proximate composition and amino acid profiles of test diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

[2] Mineral composition was tested at Midwest Laboratories (Omaha, NE, USA).

Table 7c. Performance of juvenile Pacific white shrimp *L. vannamei* (Initial weight 0.15g) offered diets formulated to evaluate *Ulva* meal as a replacement for soybean meal and fish meal on a iso-nitrogen basis in juvenile shrimp (Trial 4).

Diet	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_4D_1					
T_4D_2					
T_4D_3					
T_4D_4					
T_4D_5					
PSE[1]					
P-value					

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight-initial weight)/initial weight*100%.
[4] PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 7d. Proximate composition of shrimp at the conclusion of a 6-week growth trial in which shrimp were offered diets offered diets formulated to evaluate *Ulva* meal as a replacement for soybean meal and fish meal on a iso-nitrogen basis in juvenile shrimp (Trial 3).

Diet	Ulva Levels (%)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude fiber	Ash
T_4D_1						
T_4D_2						
T_4D_3						
T_4D_4						
T_4D_5						
P-value						
PSE[1]						

[1] Pooled standard error.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 8a. Composition of reference diet for the determination of digestibility coefficients of *Ulva* meal.

Ingredients	g/100g as is
Menhaden fish meal[2]	10.0
Soybean meal[1]	32.5
Menhaden fish oil[2]	3.2
Whole wheat[3]	47.6
Trace mineral premix[4]	0.5
Vitamin premix w/o choline[5]	1.8
Choline cloride[5]	0.2
Stay C 250 mg/kg[6]	0.1
Corn starch[3]	1.0
Lecethin[7]	1.0
Chromic oxide	1.0

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.Alabama.
[2] Omega Protein Inc., Houston TX, USA.
[3] MP Biomedicals Inc., Solon, Ohio, USA
[4]Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[5] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[6] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ,USA.
[7] The Solae Company, St. Louis, MO, USA.

Table 8b. Apparent dry matter (ADM), apparent energy (AED) and apparent protein (APD) digestibility valu ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp (*L. vannamei*).

Means	ADMD	AEDD	APDD	ADMDI	AED
Basal diet 1	76.38 ± 0.37	82.65 ± 1.20	92.08 ± 0.55		
Soybean meal	77.02 ± 0.87	82.63 ± 1.05	94.76 ± 0.49	78.51 ± 2.89	82.56 ±
Fish meal 1	68.21 ± 3.80	78.31 ± 3.21	80.86 ± 1.80	49.15 ± 12.67	69.77 ±
Ulva meal 1	62.19 ± 1.26	71.96 ± 0.89	75.14 ± 1.19	29.10 ± 4.19	40.39 ±
Basal diet 2	75.69 ±0.52	81.51 ± 0.41	92.04 ± 0.03		
Fish meal 2	67.99 ± 0.17	76.44 ± 0.78	82.34 ± 0.31	49.45 ± 0.56	65.78 ±
Ulva meal 2	64.63 ± 1.08	69.99 ± 0.64	78.33 ±0.42	38.26 ± 3.61	19.11 ±

Table 8c. Apparent amino acids (AA) digestibility value for the soybean meal (SBM), fish meal (FM), *Ulva* meal 1 (UM1) and *Ulva* meal 2 (UM2) using 70:30 replacement technique offered to Pacific white shrimp (*L. vannamei*).

AA[1]	SBM	FM	UM1	UM2
Alanine	93.75 ± 2.02	69.09 ± 4.09	36.90 ± 6.56	50.77 ± 1.10
Arginine	96.91 ± 1.44	75.35 ± 3.78	42.20 ± 6.60	47.21 ± 0.77
Aspartic Acid	95.39 ± 1.36	69.23 ± 3.70	35.87 ± 5.69	38.09 ± 1.70
Cysteine	91.29 ± 1.68	54.39 ± 7.06	13.44 ± 10.85	6.66 ± 7.45
Glutamic Acid	95.69 ± 1.52	70.84 ± 3.70	33.85 ± 7.24	23.25 ± 3.17
Glycine	95.06 ± 2.05	66.55 ± 6.26	29.84 ± 8.78	34.04 ± 4.96
Histidine	94.33 ± 1.69	74.26 ± 2.86	7.10 ± 1.87	43.52 ± 0.22
Isoleucine	93.23 ± 1.72	68.72 ± 3.99	39.15 ± 5.74	46.33 ± 0.79
Leucine	92.23 ± 1.96	71.29 ± 3.16	34.65 ± 8.50	50.43 ± 0.80
Lysine	95.03 ± 1.84	76.97 ± 2.24	40.65 ± 6.50	38.07 ± 3.04
Methionine	95.20 ± 1.54	70.63 ± 3.30	44.13 ± 5.12	40.89 ± 3.18
Phenylalanine	93.41 ± 1.90	65.28 ± 4.13	27.23 ± 7.02	47.25 ± 0.76
Proline	94.68 ± 1.92	67.21 ± 5.39	15.81 ± 10.45	18.20 ± 2.42
Serine	93.11 ± 1.91	58.31 ± 4.65	10.76 ± 11.00	43.41 ± 0.82
Threonine	91.99 ± 1.94	66.33 ± 3.35	32.83 ± 6.84	42.57 ± 0.26
Tryptophan	95.37 ± 1.92	80.31 ± 1.53	65.58 ± 2.46	70.84 ± 3.26
Tyrosine	95.28 ± 1.22	73.62 ± 3.40	36.51 ± 4.10	59.02 ± 0.45
Valine	90.78 ± 2.39	67.06 ± 3.75	29.94 ± 6.89	54.20 ± 0.42
Total AA	94.31 ± 1.67	69.91 ± 3.89	29.80 ± 6.68	41.67 ± 0.51

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